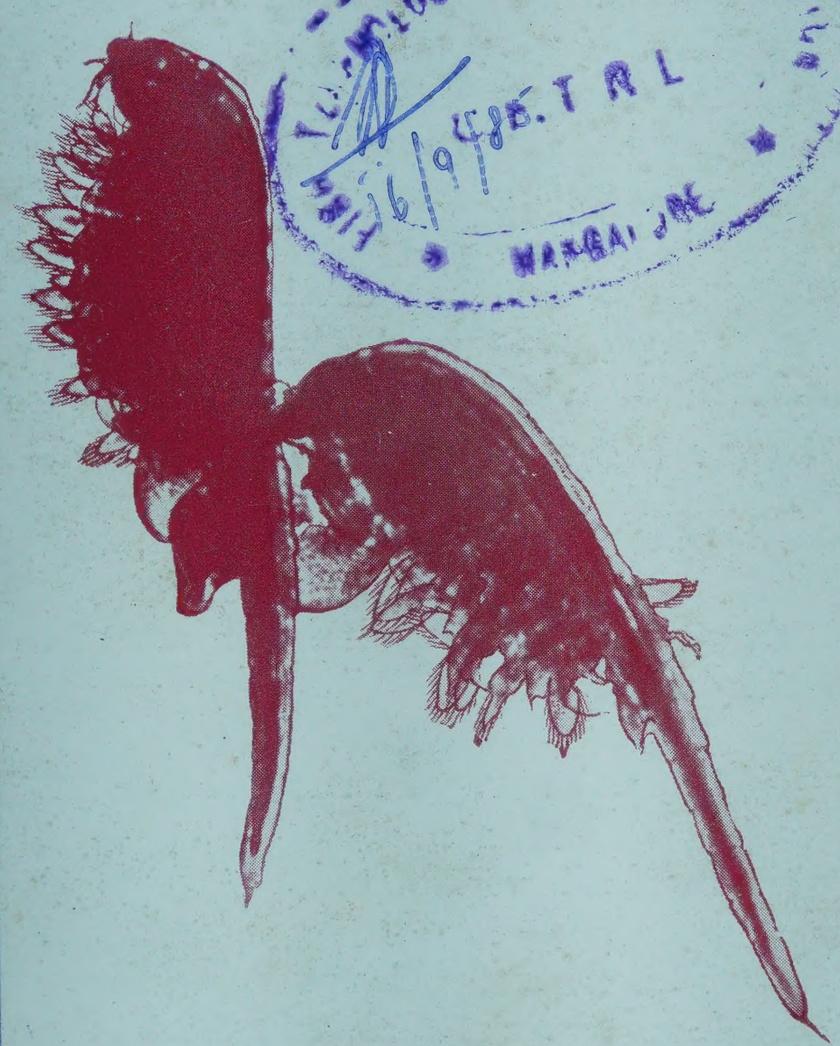
CMFRI SPECIAL PUBLICATION
Number 15

PRODUCTION AND USE OF *ARTEMIA* IN AQUACULTURE



ISSUED ON THE OCCASION OF THE WORKSHOP ON
CULTURE OF LIVE FEED ORGANISMS WITH SPECIAL REFERENCE TO *ARTEMIA* CULTURE
ORGANISED BY
THE CENTRE OF ADVANCED STUDIES IN MARICULTURE
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
AT COCHIN ON 24 AND 25 JANUARY 1984

The CENTRE OF ADVANCED STUDIES IN MARICULTURE was started in 1979 at the Central Marine Fisheries Research Institute, Cochin. This is one of the Sub-projects of the ICAR/UNDP project on 'Post-graduate Agricultural Education and Research.' The main objective of the CAS in Mariculture is to catalyse research and education in mariculture which forms a definite means and prospective sector to augment fish production of the country. The main functions of the Centre are to:

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— enhance the competence of professional staff;

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— undertake collaboration programmes; and

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Front cover : *Artemia* couple in so called 'riding position'.

Back cover : A collection of *Artemia*.

PRODUCTION AND USE OF *ARTEMIA* IN AQUACULTURE

PREPARED BY

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AND

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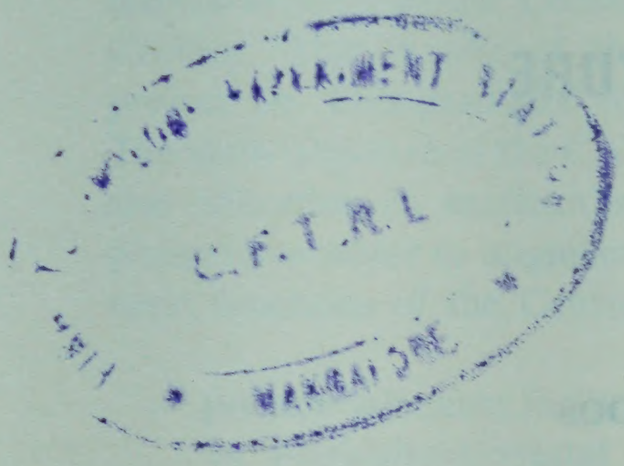

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PREFACE

For the successful culture operations of finfishes and shellfishes, feeding the larval, juvenile and adult stages with appropriate, nutritionally balanced, non-polluting, economically viable and readily acceptable feed to obtain the optimum growth and survival, is considered as one of the major requirements in aquaculture practices the world over. Live feed organisms play an important role in the dietary regime of cultivable fishes and shellfishes, particularly in the larval stages, as one or the other live feed organisms form the principal food of the larvae in nature. Culture of live feed organisms is identified as an important field as it is one of the major inputs in the hatchery seed production. Realising this, intensive investigations in the selection and large scale culture of several live feed organisms, are being carried out in aquaculture research and development programmes. Existing literature reveals that only a few species have been utilized as good live feed organisms and among them, the brine shrimp *Artemia* comes in for prime consideration.

Identifying the constraints in the promotion of live feed culture in India, the lack of appropriate technologies for sustained production on large scale, trained culturists and standard publications providing research methods and techniques stand out. In order to update our knowledge, the Centre of Advanced Studies in Mariculture, under its programme on consultancy, invited Dr. Patrick Sorgeloos, Artemia Co-ordinator, Laboratory for Mariculture, State University of Ghent, Ghent, Belgium to visit CMFRI, Cochin. During his consultancy period, a workshop on 'Culture of live feed organisms – with special reference to *Artemia* culture' was organised under his leadership and the present publication prepared by him and Dr. S. Kulasekarapandian of this Institute, was issued in dratt form. Since then it has been edited and is being published and issued in the Institute's Special Publication Series.

In this Special Publication, an attempt is made to give an update about *Artemia* culture for its cysts and biomass production. The basic research methods employed in *Artemia* culture which are particularly suitable for the tropical environment are critically discussed. Much needed informations on various facets of *Artemia* culture are provided which I hope will be helpful to research scholars and young scientists and the aquaculture industry to take up seed production more confidently. Biomass culture of *Artemia* as food for nursery reared finfish and prawn is a new concept and should prove very effective.

I take this opportunity to express my gratitude to Dr. Patrick Sorgeloos for his contribution in this publication and his co-operation for successful completion of the consultancy programme. I also express my thanks to Dr. S. Kulasekarapandian who acted as the counterpart to Dr. Patrick Sorgeloos and was intimately associated in the preparation of this publication. My thanks are also due to Dr. P. Vedavyasa Rao, Shri M. S. Muthu and Shri K. Rengarajan and other colleagues for the co-operation extended by them.

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Cochin – 682 018,
June 1984.

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1

BIOLOGY AND ECOLOGY OF *ARTEMIA*
GENERAL INTRODUCTION

1.1 Systematic Classification

Phylum	:	Arthropoda
Class	:	Crustacea
Subclass	:	Branchiopoda
Order	:	Anostraca
Family	:	Artemiidae
Genus	:	*Artemia* Leach, 1819

Among the bisexual strains of *Artemia* 6 sibling species have been described so far :

Artemia salina	:	England (now extinct)
Artemia tunisiana	:	Europe
Artemia franciscana	:	America (North, Central and South)
Artemia monica	:	Mono Lake (California–USA)
Artemia persimilis	:	Argentina
Artemia urmiana	:	Iran

Several parthenogenetical strains are found in Europe and Asia. They have important genetical differences (*e.g.* ploidy level and isoenzyme pattern) which makes their joint classification under the species designation *"Artemia parthenogenetica"* confusing. In

this regard it is suggested that unless the exact sibling species of a bisexual strain can be identified (through cross-breeding tests with known sibling species) and until specification in brine shrimp is more clearly understood especially in parthenogenetical *Artemia*, only the genus designation *'Artemia'* should be used.

1.2 Biology

Artemia can be stored "on the shelf" under the form of apparently inert particles, *i.e.* the inactive dry embryos or cysts (about 300 microns in diameter) which remain in diapause as long as they are kept dry and/or under anaerobic conditions.

Upon immersion in seawater, the biconcave cysts hydrate, become spherical and within the shell the embryonic metabolism is resumed. After about 24 hrs the cyst shell bursts (=breaking stage or E-1) and the embryo appears, surrounded by the hatching membrane (Pl. I A). Within a few hours, the embryo leaves the cyst shell completely and hangs underneath the empty cyst shell to which it is still attached (=umbrella stage or E-2, Pl. I B). Inside the hatching membrane the development of the nauplius is completed, its appendages start moving and within a short period of time the hatching membrane is ruptured and the free-swimming nauplius is born (Pl. I B).

The first instar larva which measures 400 to 500 microns in length is coloured brownish-orange and has 3 pairs of appendages: small sensorial antennulae (also called first antennae), well-developed antennae (also called second antennae) that have a locomotory as well as a filter-feeding function and rudimentary mandibles. An unpaired red ocellus or nauplius eye is situated in the head region between the first antennae. The ventral side of the head (mouth region) is covered by a large labrum. In this instar I stage no food is being taken up since the animal's digestive system is not functional yet (mouth and anus still closed).

After about 12 hrs the animal moults into the 2nd larval stage (also called instar II). Small food particles (*e.g.* algal cells, bacteria, detritus) ranging in size from 1 to 40 microns, that are filtered out

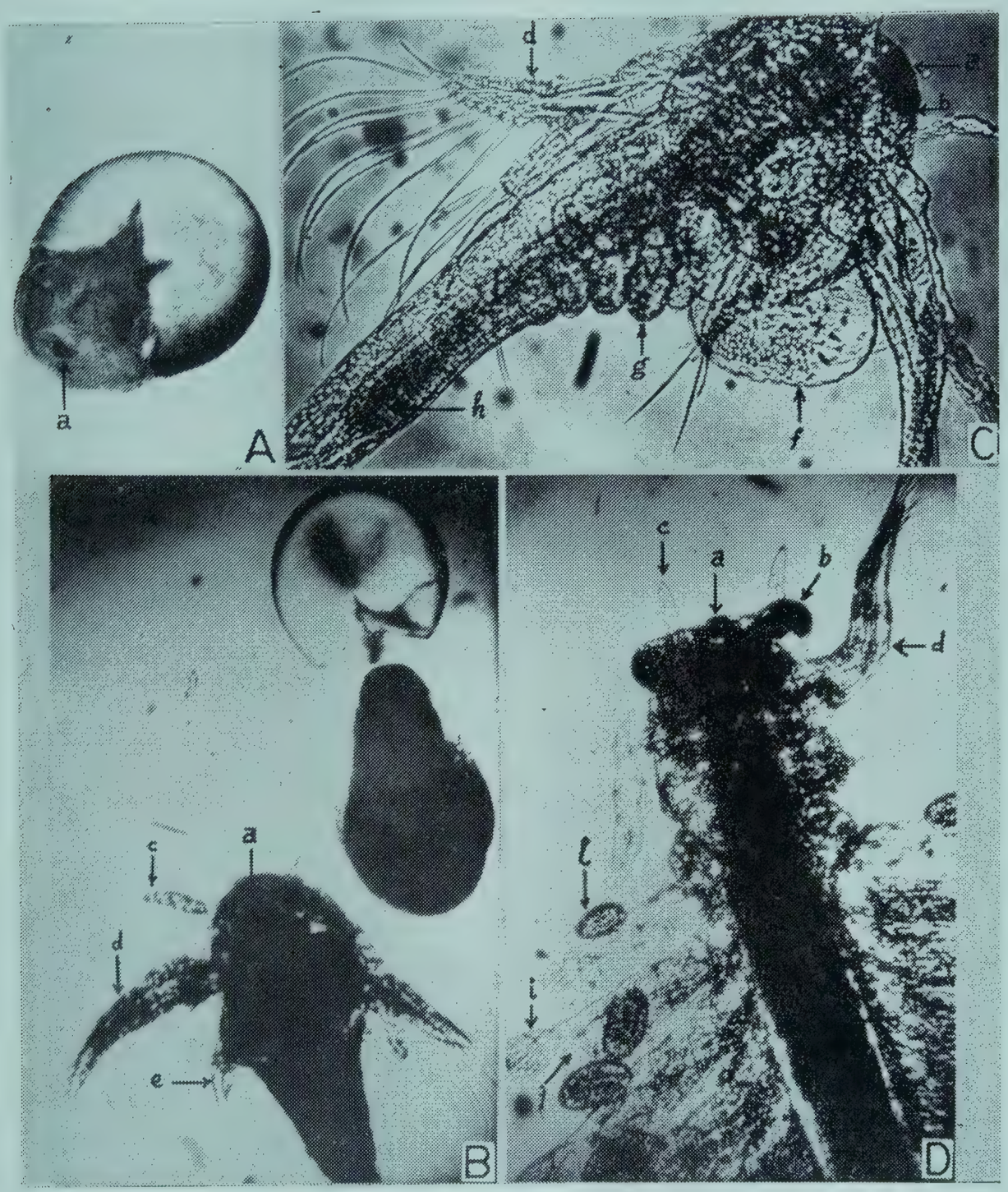

PLATE I. A. Pre-nauplius in E-1 stage; B. Pre-nauplius in E-2 stage and freshly hatched instar I nauplius; C. Instar V larva and D. Head and anterior thoracic region of instar XII (a. nauplius eye; b. lateral complex eye; c. antennula; d. antenna; e. mandible; f. labrum; g. budding of thoracopods; h. digestive tract; i. telopodite; j. endopodite and l. exopodite).

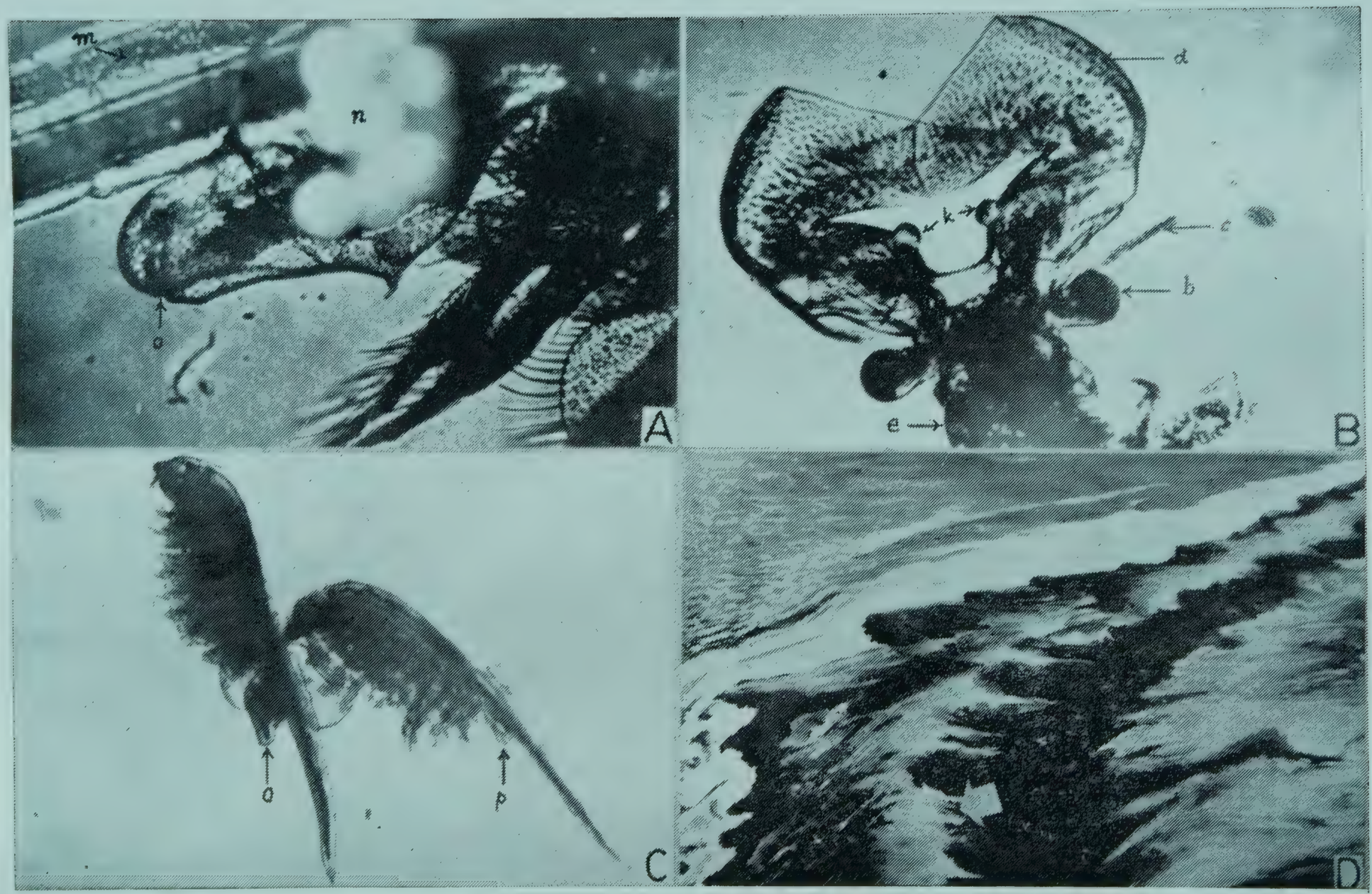

PLATE II. A. Posterior thoracic region and uterus of fertile female; B. Head of adult male; C. *Artemia* couple in riding position and D. Brown layer of brine shrimp cysts accumulated on the shore of a salina (a. nauplius eye; b. lateral complex eye; c. antennula; d. antenna; e. mandible; k. frontal knob; m. inactive ovary; n. ripe eggs in oviduct; o. uterus and p. penis).

by the second antennae are now being ingested into the functional digestive tract.

The *Artemia* grow and differentiate through about 15 moults *i.e.* the trunk and abdomen elongate, paired lobular appendages are budding in the trunk-region (Pl. I C) and will develop into functional thoracopods (Pl. I D), lateral complex eyes are developing on both sides of the nauplius eye (Pl. I C, D).

From the 10th larval stage onwards important morphological as well as functional changes are taking place *i.e.* the thoracopods are now being differentiated for locomotory, respiratory (gills) and filter-feeding functions (Pl. I D) and the 2nd antennae loose their primitive locomotory function to undergo sexual differentiation. In the males (Pl. II B) the 2nd antennae develop into hooked claspers which will become functional during copulation, while in the females the antennae degenerate into sensorial appendages.

The adult *Artemia* measures about 10 mm in the bisexual strains and upto 20 mm in some polyploid parthenogenetical strains. It is characterized by an elongated body with 2 stalked complex eyes in the head region (Pl. II B), 11 pairs of thoracic appendages and an abdomen that ends in a furca covered with spines (Pl. II C).

Precopulation in adult *Artemia* is initiated by the male in grasping the female between the uterus and the last pair of thoracopods with its muscular claspers that can open and close (Pl. II C). The couples can swim around in this so called "riding position" for long periods of time, beating their thoracopods in a synchronized fashion.

The eggs develop in paired ovaries, situated on both sides of the digestive tract behind the thoracopods. Once ripe (=spherical structure) the oocytes are being transferred via the oviducts into the unpaired broodsac or uterus (Pl. II A). At that moment copulation takes place *i.e.* the male abdomen is bent forward, one penis (the male *Artemia* has 2 organs) is introduced into the uterus aperture and sperm is being released.

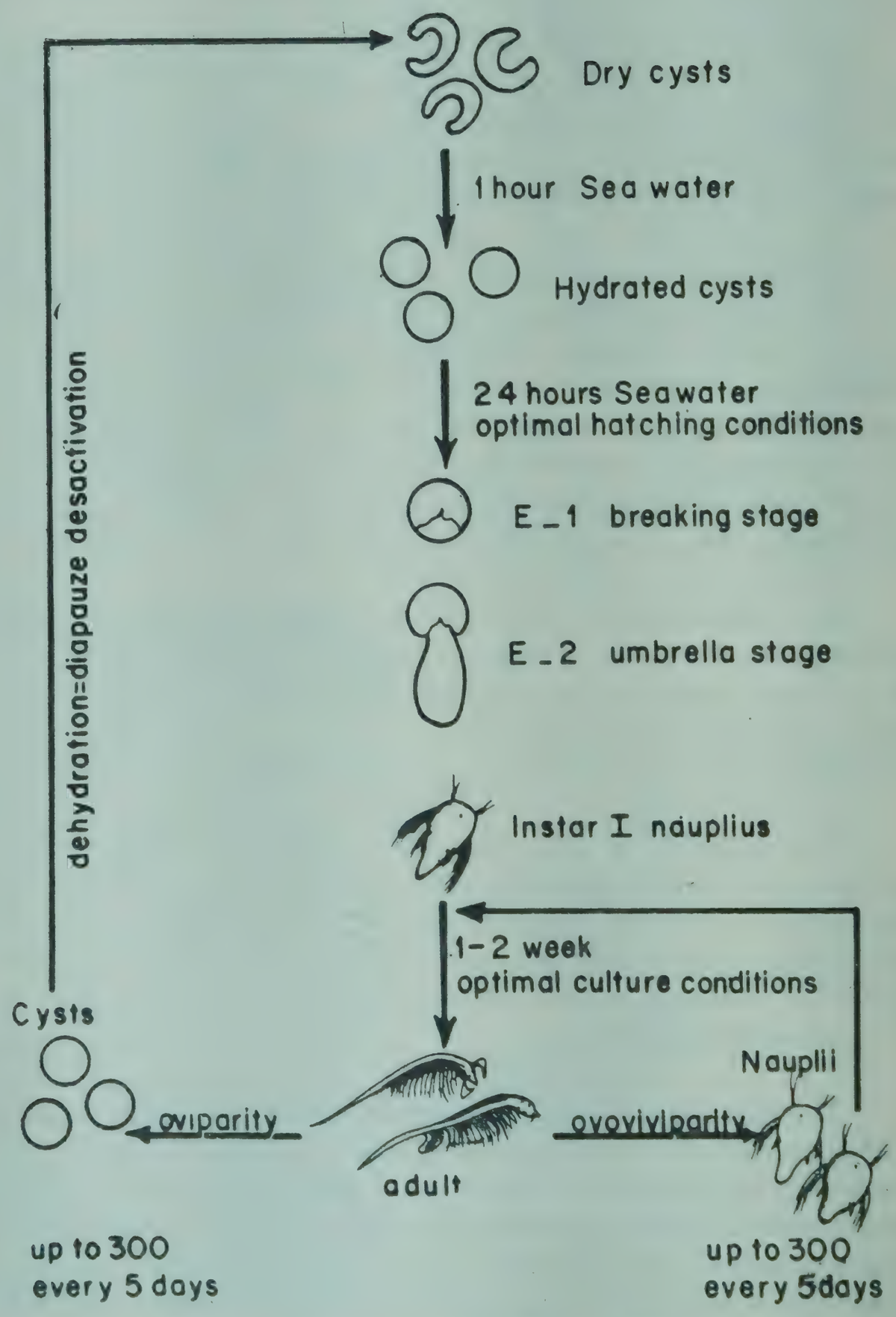

Fig. 1. Schematic diagram of *Artemia* life cycle.

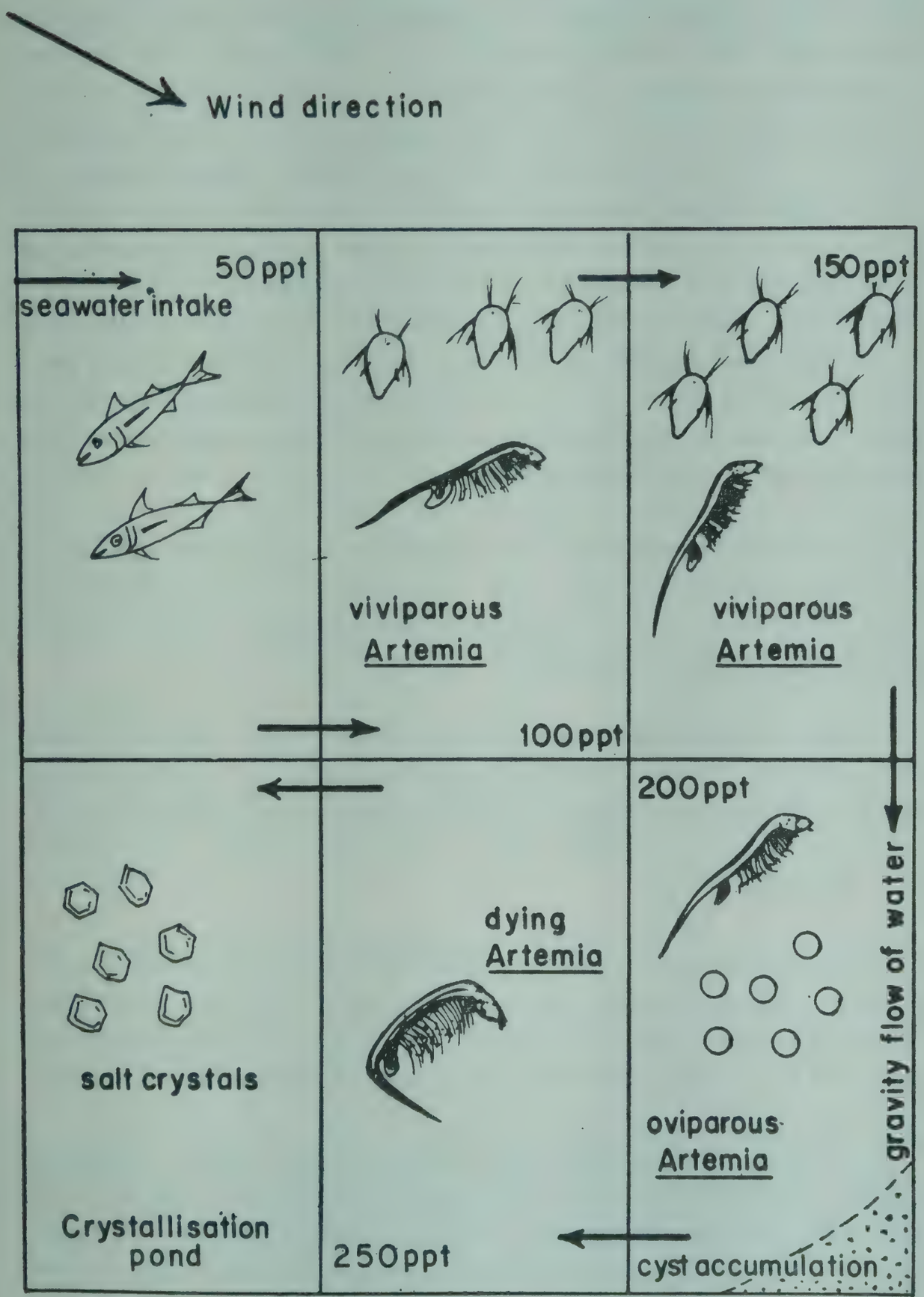

Fig. 2. Schematic diagram of solar salt operation with natural occurrence of *Artemia*.

The fertilized eggs normally develop into free-swimming nauplii (=ovoviviparous mode of reproduction) which are set free by the mother. In extreme conditions (*e.g.* high salinity, low oxygen levels or food shortage) shell glands (*i.e.* grape like organs located in the uterus) become active and accumulate a brown secretion product. The embryos only develop upto the gastrula stage at which moment they are surrounded by a thick shell or chorion (that is secreted by the brown shell glands), enter a state of dormancy or diapause and are deposited (=oviparous mode of reproduction). The latter cysts usually float and are blown ashore where they accumulate and dry (Pl. II D). As a result of this dehydration process the diapause mechanism is inactivated allowing the cyst to resume its further embryonic development when hydrated in seawater of sufficiently low salinity.

The same reproductive characteristics are valid for parthenogenetical *Artemia* with the only exception that fertilisation has not to take place and the embryonic development starts as soon as the eggs reach the uterus.

Brine shrimp can live for several months, grow from nauplius to adult in less than 2 weeks time and reproduce at a rate of upto 300 nauplii or cysts every 5 days (see schematic diagram in Fig. 1).

1.3 Ecology

Brine shrimp thrive very well in natural seawater but do not possess any anatomical defense mechanism against predation; consequently they are always in danger at salinities which are tolerated by carnivorous species (*e.g.* fish, crustaceans and insects).

Artemia, however, have developed a very efficient ecological defense mechanism by their physiological adaptation to media with very high salinity, where their predators cannot survive. For this they possess the best osmoregulation system known in the animal kingdom; in addition they are capable to synthesize very efficient respiratory pigments or haemoglobins to cope with the low oxygen levels that prevail at high salinity; and finally they have the ability

to produce dormant cysts which can resist to extreme environmental conditions when juveniles as well as adults are wiped out.

Artemia are found in natural salt lakes as well as in man-made salterns. Different geographical strains have adapted to widely fluctuating conditions with regard to the temperature (6 – 35°C) and the ionic composition of the medium (chloride, sulphate as well as carbonate rich waters).

Artemia feed on particulate matter of biological origin *(e.g.* organic detritus from mangrove waters) as well as on living organisms of the appropriate size range (microscopic algae and bacteria). The presence of algal material or other particles in the intestine of brine shrimp should not be considered as an evidence of its nutritional value nor of its digestibility; in fact, brine shrimp are non-selective filter-feeders that ingest anything in the size-range of 1 to about 50 microns.

At salinity above 100 ppt *Artemia* do not have food competitors (the larvae of the brine fly *Ephydra* are benthic feeders) and often develop into large monocultures the densities of which are mostly controlled by food limitation. Ovoviviparous reproduction is mostly dominant at low salinity levels, whereas cysts mostly are produced at salinity beyond 150 ppt (see schematic diagram in Fig. 2).

1.4 Geographic Distribution

So far over 300 natural *Artemia*-biotopes, spread over the 5 continents have been identified.

Wind as well as waterbirds (especially flamingos) are considered to be the most important natural dispersion vectors. Nonetheless, in recent times man has been responsible for several *Artemia* transplantation in S. America and Australia either for salt improvement or for aquaculture production purposes.

The distribution of *Artemia* is limited to biotopes where salinity always are sufficiently high to keep out predators or where

low temperatures during winter time (when it rains) assure the ametabolic state of the hydrated cysts. Climates with a water-surplus, *e.g.* with distinct dry and wet season might provide suitable conditions for *Artemia* occurrence during the dry season (*e.g.* thousands of hectares of solar saltworks in S.E. Asia), however, the brine shrimp population could not withstand predation during the rainy season.

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2

EXPLOITATION OF *ARTEMIA* FROM NATURAL HABITATS

2.1 Introduction

Natural populations of *Artemia* are found in salt lakes (coastal or inland; chlorine, sulphate or carbonate rich waters) and especially on coastal salinas (man-made and/or man-managed solar saltworks). *Artemia* is only occurring in the evaporation ponds at intermediate salinity levels *i.e.* from about 100 ppt onwards (when predators have been eliminated by salinity stress) upto about 200 – 250 ppt (when food becomes limited for *Artemia, i.e.* higher energy consumption as a result of increased osmoregulatory activity).

Provided the intake waters of the saltwork are rich in nutrients, *Artemia* can develop into dense populations. Depending on the local strain as well as the physical-biological conditions in the ponds (*e.g.* water retention time, water depth, pond productivity, etc.) cysts are eventually produced (seasonally or year round) and driven by the wind, accumulate on the shore of the evaporation pond. At still higher salinities the *Artemia* die off and are completely metabolized by the bacterial flora.

The NaCl-salt which finally precipitates in the crystallizer ponds is not contaminated what so ever by *Artemia*. On the contrary,

it has been proven in different solar saltworks that the presence of brine shrimp favour the salt production qualitatively as well as quantitatively; *i.e.* (1) efficient removal of the planktonic algae by *Artemia* assures gypsum ($CaSO_4$) – precipitation early in the brine flow (and thus not in the crystallizers where it contaminates the salt), (2) salt crystals will not contain organic inclusions (impurities) as particle matter has been efficiently removed by the filter-feeding brine shrimp and (3) finally, *Artemia*-metabolites as well as decaying *Artemia* will be used as a useful substrate by the halophilic bacteria of the genus *Halobacterium* which develop in the crystallizers and colour the water dark-red, thus assuring better heat-absorption, increased water temperatures and as a result faster salt precipitation.

As saltworks are mostly operated by (chemical) engineers, locally occurring brine shrimp are either not observed or it is not known that the natural *Artemia* can be valorized as a valuable by-product. More and more salt operations are now developing an *Artemia* business-line: either by selling cysts and/or biomass (live or frozen) to the aquarium petmarket and to aquaculture industries, or they set up a vertically integrated aquaculture project, taking advantage of the various *Artemia* products in a nearby fish or shrimp farm.

2.2 Harvesting of Cysts

* Cysts should be harvested as soon as possible after production (accumulation), preferentially in the morning:

–cysts with a pale colour (=low content of haematine in chorion) are not well protected (embryo viability) against the UV radiation from the sun;

–cysts might dry up on the shore and eventually be carried away by the wind;

–cysts that accumulate on the shore might be exposed to repeated hydration/dehydration cycles (rainfall, high humidity) and loose their energy reserves (resulting in decreased hatchability or reduced caloric contents).

*Cysts should preferentially be harvested from the water (surface), not from the shore, thus assuring less contamination with impurities and reducing chances for quality decrease (see above). Therefore it is advisable either to make steep dikes or to install cyst barriers close to the shoreline but in the water (Fig. 3).

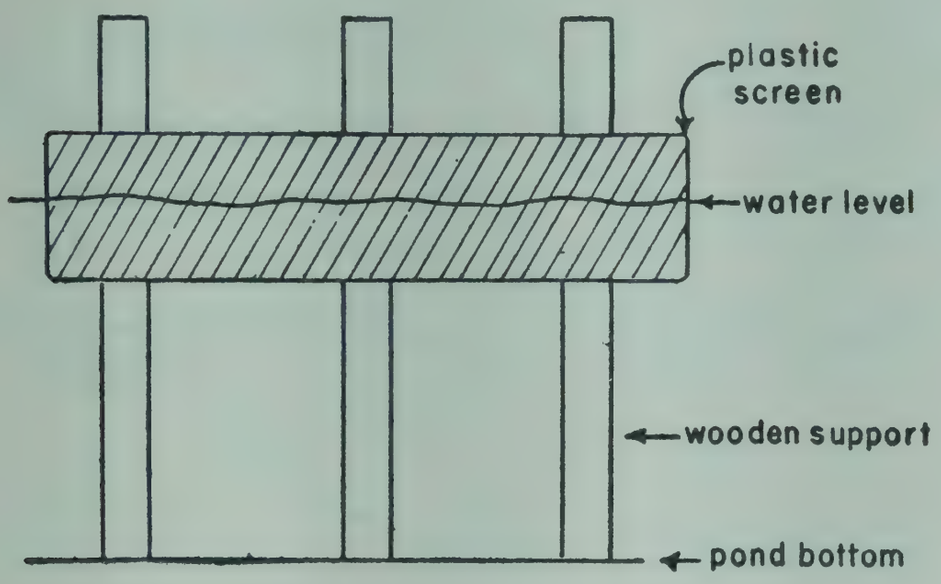

Fig. 3. Cyst barrier.

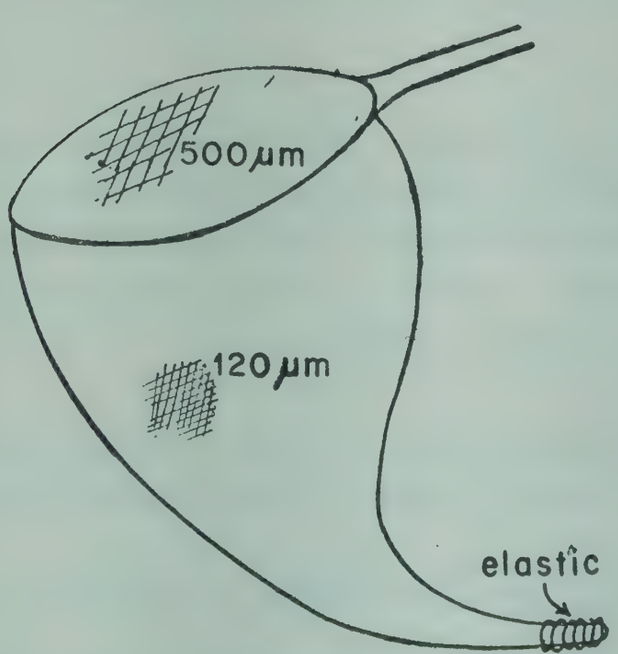

Fig. 4. Double screen dip net.

*Collect cysts with double-screen dipnet (500 μ m screen to remove adults; 120 μ m screen to collect cysts) (Fig. 4).

*When much foam is being produced in which cysts normally get trapped and lost (foam gets airborne), wave-breakers should be installed in 2 or more rows (at about 1 m distance from each other) parallel with the cyst barrier; use bamboo mounted on poles just underneath water surface (Fig. 5).

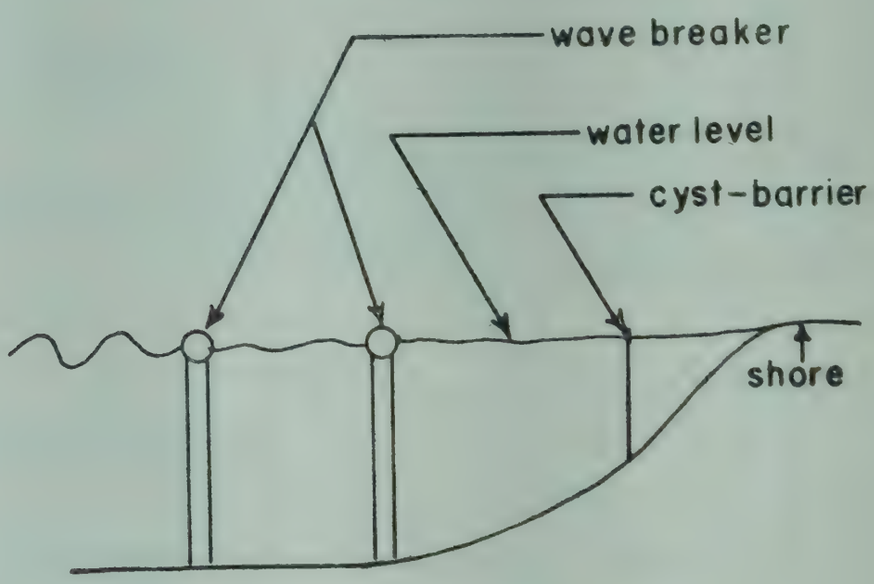

Fig. 5. Reduction of foam formation by installing wave breakers.

* Harvested cysts should be stored in a closed container in saturated brine (solar salt); it is advisable to regularly (*e.g.* once a day) stir up the floating cysts as to assure that all cysts are properly dehydrated; assure continuous presence of salt crystals at bottom of tank (guaantee for saturated brine).

* Cysts should be processed (further cleaning and drying) after not more than one month storage in the saturated brine container.

2.3 Harvesting and Processing of Biomass

Adult *Artemia* can be manually harvested with a dip-net. In most salinas, however, brine flows by gravity from one pond into another which allows automatic harvesting *i.e.* large nets are installed in the canal that connects 2 ponds and *Artemia* are retained from the brine that is being drained into the adjacent pond (Fig. 6).

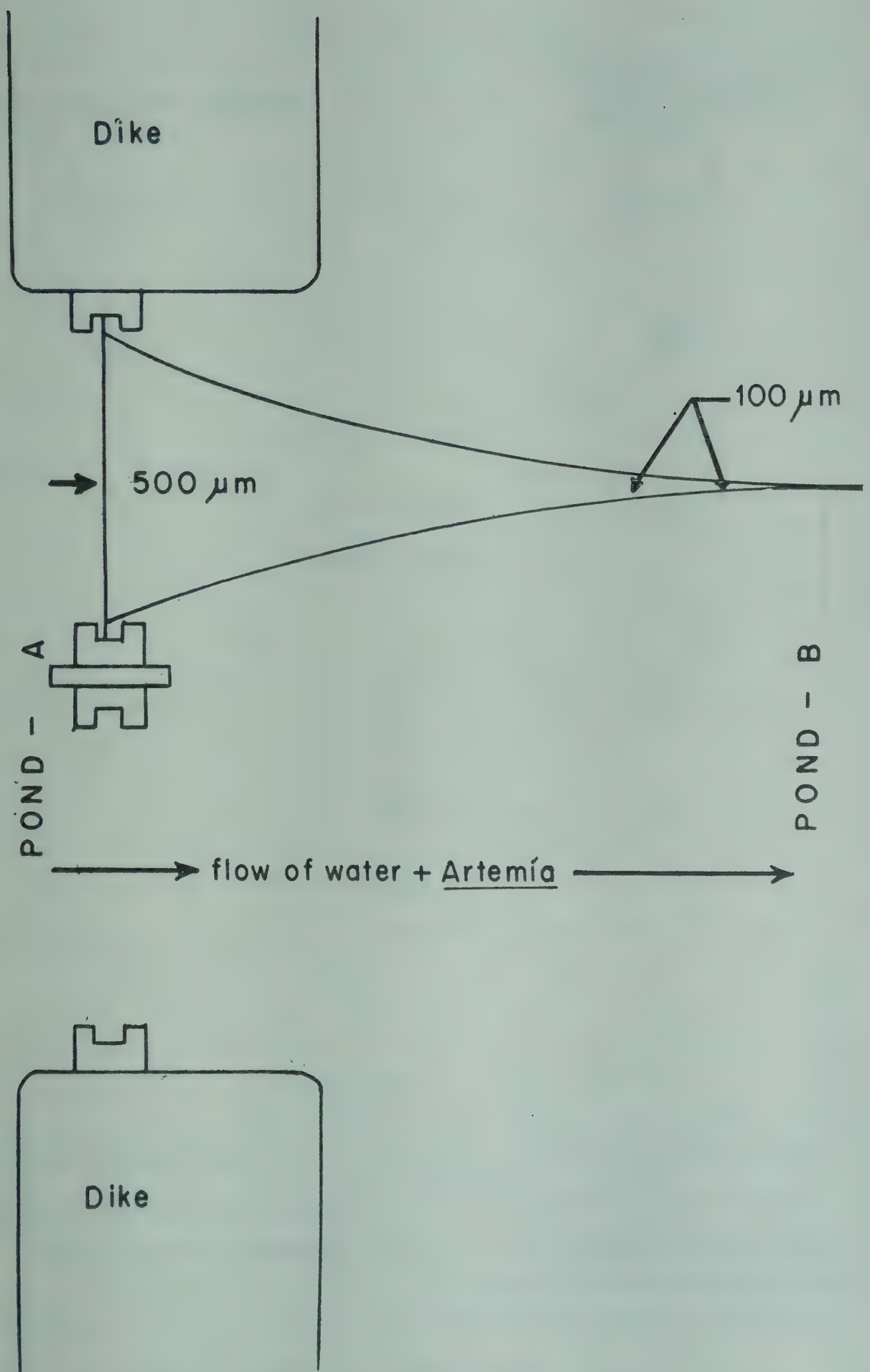

Fig. 6. *Artemia* harvest.

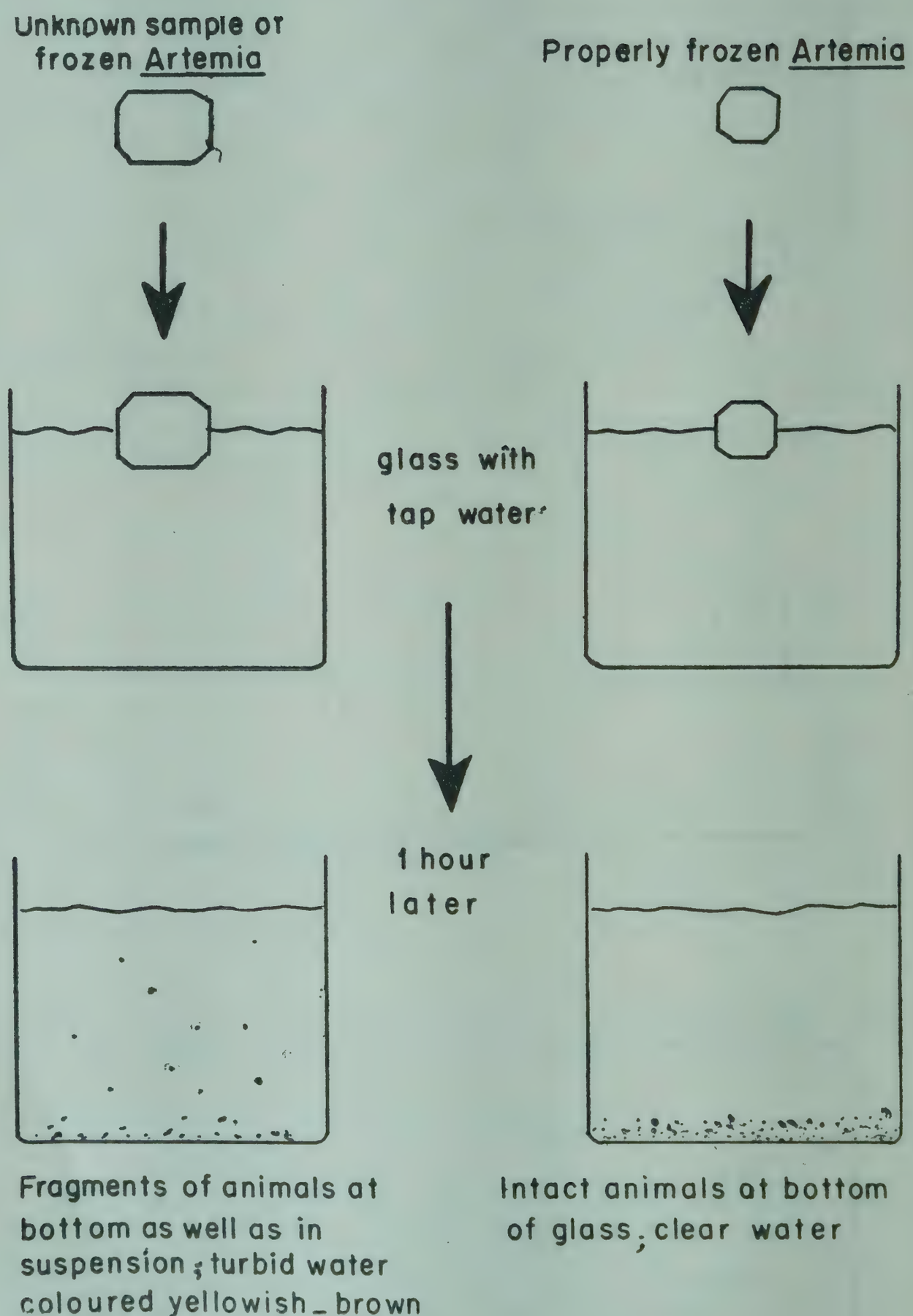
Unknown sample of frozen Artemia
Properly frozen Artemia
glass with tap water
1 hour later
Fragments of animals at bottom as well as in suspension; turbid water coloured yellowish-brown
Intact animals at bottom of glass, clear water

Fig. 7. Verifying the quality of frozen *Artemia*.

Nets should be quite large as to facilitate harvesting; *e.g.* for harvesting over 100 kg of adult biomass per hour the filter-diamensions should be as follows: filter-mouth of 1.5 by 1 m and filter-length of about 3 m.

The end-part of the net where the adult *Artemia* accumulate should have a small mesh size (less than 100 μm) as to prevent extrusion of the animals.

Nets should be emptied at minimum 1 hour intervals : *i.e.* *Artemia* that accumulate at the end of the filtersac are exposed to anaerobic conditions which they can tolerate for not more than 2 hours; since *Artemia* is rich in proteolytic enzymes it is essential to harvest it alive.

For direct (live) feeding of the harvested *Artemia* to marine as well as freshwater animals it is sufficient to excessively wash the animals as to remove all (inter-animal) brine; in fact since *Artemia* is a hypo-osmoregulator - its body fluids are always at about 9 ppt even when collected from a 180 ppt evaporation pond.

Adult *Artemia* harvested from a wild population (living at salinities of minimum 100 ppt) will not survive the severe salinity shock when transferred to natural seawater, however, they will remain alive (even when put in freshwater) for at least another 3 to 5 hours, during which time they should have been eaten by the predator.

In order to assure optimal product quality, *Artemia* must be frozen when still alive. The live biomass should be spread out in thin layers, *e.g.* maximum 1 cm thick layer in plastic bag, or in ice-trays (small cubes) and be transferred to a quick freezer (at least - 25°C); this way the animals remain intact and do not loose their body fluids when being thawed. The quality of frozen *Artemia* can easily be verified shown as in Fig. 7.

The nutritional quality of *Artemia* is greatly reduced when drying the biomass in the sun or an oven. Only the cost-prohibitive technique of lyophylisation assures quality maintenance of lipids, proteins, vitamins, etc.

2.4 Bibliography

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3

CYST PROCESSING

3.1 Introduction

The quality of *Artemia* cysts mainly depends on the way the cysts are processed and stored. The *Artemia* cysts, collected from ponds, may be contaminated with sand particles, dirt material, dead *Artemia,* algae, debris, empty shells, broken shell bits, plumes, etc. and the quality of the end product (hatching efficiency) is largely determined by the effective removal of all these dirt materials from the cysts. In addition to purity, cyst water content is an important criteria to mark the quality of the cyst and high quality cysts will have very low water content (less than 10%). Hence the cleaned cysts must have been adequately dehydrated.

3.2 Processing

3.2.1 Material

Cysts of the Great Salt Lake *Artemia* strain.

3.2.2 Equipment required

100 μ m and 400 μ m sieves, brine, drying plate, hot air oven, vacuum pump and siphon tube.

3.2.3 Procedure

Wash the raw cysts rapidly with freshwater by passing through 400 μ m sieve and collect them on a 100 μ m sieve, to remove larger particles (larger than 400 μ m in size).

Wash within 5 minutes with freshwater while the cysts are retained on 100 μ m sieve.

The cysts, subsequently, have to be transferred to brine solution where heavy debris will sink to the bottom.

Stir the cysts in brine by aeration (by keeping the airtube 5 cm below the cyst level) or manually by means of a glass rod, in order to facilitate better separation.

Siphon out the floating cysts in brine on a 100 μ m screen.

Transfer the cysts to freshwater in which full cysts will sink and light debris/empty shells will float.

Stir manually or with aeration as to assure better separation from debris.

Retain in freshwater for 15 minutes.

Collect the full cysts from the bottom on (100 μ m) filter cloth bag.

Drain and squeeze out water as much as possible.

Dry the cysts in thin layers in hot air oven at 30 – 40° C.

Redistribute the layers in the oven at every hour for effective drying.

Dry the cysts until they attain a constant weight.

Store them in vacuum polythene vial for storage.

3.3 Principle of the Procedure

All the processing techniques go through the following three stages, namely cleaning, dehydration and packaging.

3.3.1 Cleaning

Cleaning can be done by washing the cysts with freshwater using different mesh-sized screens so as corresponding sized dirt materials will be removed. During washing the cysts by keeping them on 400 μ m sieve, dirt materials of above 400 μ m size will be removed from the cysts. When the cysts are retained on 100 μ m screen and washed, the debris of above 100 μ m but below 400 μ m will be found along with the cysts. Very important criteria to be observed is that the cleaning in freshwater must be very quickly done because prolonged cleaning in freshwater will initiate hydration and subsequent embryological development resulting in energy loss.

The dirt materials which are equal in size with the cysts can be removed by the biphase floatation method. At first, the cyst material is suspended in brine. In this solution, cysts and light debris will float and the heavy particles such as sand will sink to the bottom. An intermittent aeration from an air tube at a distance off the bottom improves the separation of cysts and debris. This brine separation should be continued for about 24 hour (duration not critical). The layer of floating cysts is creamed off and the cysts are thoroughly washed with tap water on a 100 μm screen. Secondly the separation of the light debris is carried out in freshwater and this treatment is only for a short period of 15 minutes (otherwise, the cysts will reach the hydration level with consequent start of metabolism). The full cysts (viable ones) will sink to the bottom whereas the empty cyst shells, plumes, etc. float at the surface. The cleaned cysts are then siphoned off in a (100 μm) cloth bag and the excess water is to be drained.

3.3.2 Dehydration

The cysts have to be spread in uniform thickness over a drying surface and kept for drying in the shade or in a hot air oven at 30 – 40°C. At every hour redistribute the cysts for effective drying. Drying should be continued until there is no further loss of weight. When there is no change in the weight during drying, it can be presumed that the water content in the cysts has reached the desirable level of 2 – 9%.

3.3.3 Storage

For storage upto a few months, the cysts need not to be dried and can be stored in vials containing clean brine. This can be done after cleaning thus omitting the air drying procedure. If storage is for a term of 6 months or a year, it is sufficient to store the air-dried cysts in closed glass or plastic vials filled to brim. As long as they are kept dry, viability will not be affected significantly over a period of one year. There is no need to keep them in the refrigerator. If storage extends over periods of more than a year, or if the cysts have to be packed for commercial purpose, it is necessary to pack them dry under vacuum or nitrogen atmosphere.

3.4 Observation

Observe that the viable cysts are floating in the brine solution and sinking in freshwater or seawater. Observe that the cysts attain the biconcave shape after complete drying.

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4

CYST QUALITY ANALYSIS

4.1 Introduction

Cyst quality can be assessed on the basis of its moisture content, hatching efficiency, hatching percentage and hatching rate besides its nutritional value. During prolonged period of cyst storage, periodical quality analysis is necessary to make sure that the cysts are retaining their viability.

4.2 Analysis

4.2.1 Materials to be analysed

Cysts of Great Salt Lake (Utah – USA), Chaplin Lake (Canada), Mossoro (Brazil) and Reference *Artemia* Cysts.

4.2.2 Equipments required

100 ml measuring cylinder, 1 ml graduated pipette, Lugol's solution, petri dish (both top and bottom – 50 mm diameter in size), binocular microscope, aeration and aluminium tarra and chemical balance.

4.2.3 Procedure

A. To find out the water content :

1. Take the weight of the aluminium tarra after keeping it at 60°C for 1 hour.....(A)

2. Take the weight of the sample (of about 500 mg) + tarra.....(B)
3. Dry it in the oven at 60°C for 24 hours (in the presence of hygroscopical material, *e.g.* silicagel, $CaCl_2$)
4. After 24 hours, take the weight of sample + tarra at 60°C.....(C)
5. Calculate the water content of the cyst by making use of the following formula

$$\text{Water content (in percentage of dry weight)} = \frac{C-A}{B-A} \times 100$$

6. Keep three such samples and take an average value for the water content.

Remarks

Drying should not be done at more than 60°C as lipid globules of the cysts will be volatized at high temperature.

B. To find out the hatching efficiency (HE):

Standard method

1. Take 80 ml of seawater in 100 ml measuring cylinder.
2. Aerate the water.
3. Add 250 mg cyst.
4. After one hour make up the volume to 100 ml by adding seawater.
5. Take five subsamples of 0.25 ml each in five petri dishes using a graduated 1 ml pipette (tip of pipette to be cut off).
6. Rinse the tip of the pipette and adjust the volume to approximately 4 ml in each petri dish by adding fresh seawater.
7. Incubate at continuous light for 48 hours.
8. Fix with one drop of Lugol's solution.
9. Count the larvae (N) per petri dish.
10. Take an average.
11. Calculate the hatching efficiency by making use of the following formula.

$$\text{Hatching efficiency} = \frac{\text{Number of larvae}}{\text{One gram of product}} = \frac{\bar{N} \times 4 \times 100 \times 4}{1}$$

Simplified method

1. Take 80 ml of seawater in 100 ml measuring cylinder; aerate and add 250 mg cysts.
2. After one hour adjust to 100 ml by adding seawater.
3. Provide continuous aeration and illumination.
4. After 48 hours, take 5 subsamples of 0.25 ml each in petri dish with 1 ml graduated pipette.
5. Wash the tip of the pipette and add to petri dish. Bring volume to 5 ml.
6. Add 1 drop of Lugol's solution.
7. Count the larvae per petri dish and make an average ($\bar{N}$).
8. Calculate the hatching efficiency (HE) by applying the following formula.

$$HE = \bar{N} \times 4 \times 100 \times 4$$

C. To find out the hatching rate (as well as hatching synchrony) :

Apply standard method or simplified method as explained above taking into account that starting after 15 hours incubation every 3 hours five samples each have to be counted.

Therefore using the standard method many more petri dishes have to be set up (40 in total to follow hatching rate from 15 to 36 hours after incubation).

It might be more practical to use the simplified method and take the subsamples from the 100 ml hatching container at 3 hours intervals.

D. To find out the hatching percentage :

Use standard method or simplified method as explained above and incubate cysts for 48 hours period.

Do not add Lugol's solution but add few drops of hypochlorite to petri dishes at the end of incubation *i.e.* chorions will dissolve and it will be possible to distinguish nauplii, unhatched embryo's as well as empty chorions.

Calculate the hatching percentage by making use of the following formula:

$$\text{Hatching percentage} = \frac{\text{Number of nauplii} \times 100}{\text{Number of nauplii} + \text{Number of unhatched embryos}}$$

4.3 Bibliography

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5

CYST HATCHING

5.1 Introduction

The principal reason why brine shrimp nauplii are so widely used for aquaculture purposes is that their culture for feeding predators can be started from an (apparently) inert source, namely dried cysts. Extensive literature exists on the hatching of *Artemia* cysts. Embryological development has already occurred upto the gastrula stage in the dormant *Artemia* cysts. Certain mechanisms are involved in the restarting of the "biological clock" in these cysts while they are subjected to hatching.

5.2 Hatching

5.2.1 Material

Cysts of the Great Salt Lake *Artemia* strain.

5.2.2 Equipments required

Conical container, source for aeration (requirement being 10–20 litre of air per minute), light source for minimum 1000 lux (Tube lights), seawater and technical grade $NaHCO_3$.

5.2.3 Procedure

Take the cysts to be hatched in conical (transparent glass or plastic) hatching container.

Add filtered (clean) seawater the required quantity being maximum of 5 grams of cysts per litre of seawater.

Supply moderate aeration from the bottom of the container at the rate of 10 to 20 litre of air per minute.

Provide light to minimum of 1000 lux or start hatching during day time. If hatching is to be started during night or late evening, provide one or two tube lights in front of the hatching container.

Take regular samples and observe the different stages of hatching under microscope.

5.2.4 Principle of the procedure

At least five conditions are essential for restarting the embryological development in cysts leading to the hatching of the nauplii. They are (i) hydration of the cysts in seawater, (ii) oxygenation of the medium, (iii) illumination of the hydrated cysts, (iv) pH above 8.0 and (v) temperature of 26–30°C. Hatching can be carried out in salinities ranging from 5 to 75‰. Instead of seawater, a solution prepared from 2 teaspoonful of common table salt dissolved in one litre of fresh water can also be used as medium for hatching small quantities of cysts (this water is not enough buffered for hatching high densities of cysts). For practical convenience, seawater (enriched with 2 gm $NaHCO_3$ per litre) is used for hatching. Oxygen content of the medium plays a vital role in hatching. It is reported that hatching rate is constant in the range of 2 to 8 ppm dissolved oxygen with the California strain. Below this value, hatching efficiency decreases and is even completely inhibited at 0.6 to 0.8 ppm. Continuous moderate aeration which keeps the cysts in suspension, is beneficial to hatching. If the cysts accumulate at the bottom, many become rapidly subjected to anaerobic conditions and this results in the embryological development being blocked. Hatching efficiency is considerably higher in light as compared to dark. Hatching experiments with cysts from Bulgaria and USA revealed that light triggers the internal "biological clock" to start again. Brief illumination of the cysts after hydration is

sufficient to assure good hatching. The minimum exposure time depends on the strain used and light intensity. Illumination for 10 minutes at an intensity of 1000 lux is sufficient for cysts of California strain. The light triggering is only effective in aerobic conditions. Optimum hatching temperature varies from race to race, for example more than 50% hatching after 36 hours for California strain at 28°C and for the Utah strain at 30°C. Thus, to obtain good hatching, the eggs must be exposed to light (at least just after hydration) in order to assure that the embryo's development is triggered; the medium must be continuously oxygenated and the eggs must be kept in suspension.

Several types of hatching containers have been used by different workers. Rectangular hatching and separator boxes are used by Shelbourne *et al.* (1963). Jones (1972) has used large flat-bottomed opaque plastic vats for hatching. In the latter hatching containers, the cysts get driven into the corners because of the flat-bottomed nature. Strong aeration is needed to maintain the suspension of the cysts in vessels with flat and large area, but it is not desirable as the freshly hatched nauplii are quite sensitive to vigorous air-bubbling. Further only small quantities ranging from 0.3 to 1.0 g cysts per litre can be effectively hatched in these type of containers. However, these problems can be solved if glass or plastic funnel shaped containers are used for hatching (Persoone and Sorgeloos, 1975). Because of the transparency, adequate illumination is ensured. As the bottom is funnel shaped, moderate aeration sufficiently aerates the medium and simultaneously keeps the cysts in adequate suspension. Addition of a few drops of a nontoxic silicone antifoamer will prevent foaming, if present. In these cylindrical containers, densities of 10 gm cysts per litre with a yield of 70% with Utah cysts and 90% with California strain have been regularly hatched.

5.2.5 Observation

Observe under microscope :

(i) the breaking of the cysts (breaking stage) after 12–24 hours;
(ii) the embryo in 'umbrella stage' after 36 hours;

(iii) the living embryo moving within the hatching membrane;
(iv) that all the nauplii hatch out within 48–72 hours;
(v) the structure of the freshly hatched nauplius and
(vi) the morphological difference between first, second and third instar stage.

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6

SEPARATION OF HATCHED NAUPLII FROM THE HATCHING DEBRIS

6.1 Introduction

It is of paramount importance that after hatching, *Artemia* nauplii have to be separated from the unhatched and empty cysts. It has been observed that consumption of empty cysts blocks the gut when fish larvae are fed with uncleaned *Artemia* nauplii. Unhatched and empty cysts have a very high bacterial load and hence care has to be taken to avoid them by separating the hatched nauplii alone.

6.2 Separation of Hatched Nauplii

6.2.1 Material

Freshly hatched nauplii of Great Salt Lake *Artemia* strain.

6.2.2 Equipment required

Nauplii separator and siphon tube.

6.2.3 Nauplii separator

The separator is a circular tank (30 cm diameter x 15 cm height) and at its centre a dark coloured cylinder (10 cm diameter x 15 cm height) is glued to the bottom thereby dividing the separator into

an inner and outer compartment. The inner compartment has several slits which connect both the compartments. It is threaded at its outer side as to allow another dark cylinder (10 cm diameter x 15 cm height) to be screwed on it. By moving the dark tube of the inner compartment, the slits will be closed or opened. If it is rotated clockwise, the slits are closed and the connection between the compartments will be cut off. The dark tube of the inner compartment can be closed off with a lid as to assure complete darkness inside this compartment. Nauplii separation can be enhanced by illuminating the outer compartment with extra light source.

6.2.4 Procedure

Take adequate water in both compartments of the separator.

Place the nauplii debris mixture in the central dark compartment.

Close the lid of the central compartment.

Open the connecting slit of the separator by slightly rotating the central compartment anti-clockwise.

Observe the nauplii moving towards the outer compartment through the slits from the inner compartment.

Wait for ten minutes.

Close the connecting slits by rotating the central compartment clockwise.

Remove the collected nauplii by siphoning the contents of the outer compartment.

Repeat the process two or three times.

6.2.5 Principle

The positive phototactic behaviour of the nauplii is exploited for separating the nauplii from the empty and unhatched cysts. Directing a light beam on the transparent hatching device results in the larvae swimming towards the light as soon as the aeration

has been turned off. They can be siphoned off from that particular place. This rough separation technique, although commonly used, has various disadvantages: (i) It is a time consuming process and it requires skill to remove the nauplii without siphoning off the debris accumulated on the bottom and surface. (ii) Separation is qualitatively and quantitatively far from being optimal. Many empty shells have exactly the same density as the medium and consequently will be siphoned off with the larvae. (iii) As the medium is not aerated during the long separation time, this system does not permit the handling of high larval densities without the risk of the nauplii suffering an oxygen shortage.

Shelbourne *et al.* (1963) and Riley (1966) have used separator boxes in which, the nauplii, hatched in darkness, swim through holes or slits from the dark compartment (hatching tank) to the brighter side (separator tank). Once separation is completed, the partition can be closed and the larve siphoned off. In these rectangular separator boxes, separation is rather poor due to the tendency of the nauplii being too far from the illuminated area to receive the phototactic stimulus; furthermore, hatching is not optimal as the cysts are not exposed to light.

These problems can be solved by using a cylindrical separator box. Using this apparatus, the mixture of nauplii and debris is introduced in the closed central dark compartment. When the connecting slits are opened by slightly rotating the inner compartment anticlockwise, the nauplii move through the slits from the dark inner compartment towards the brighter outer compartment. After about 10 minutes, the connection can be cut off and the collected nauplii can be removed from the outer compartment. As the nauplii are kept in the separator for a short period, they will not suffer from oxygen deficiency. Separation is quick and there is absolutely no chance for the subsequent mixing of the nauplii (moved towards the outer compartment) with the empty shells (which are retained in the central compartment). As the separator is cylindrical, the light stimulus is uniform for all the nauplii from any direction.

6.2.6 Observation

Observe that nauplii move towards the brighter outer compartment through the slits from the dark inner compartment thereby separating themselves from the debris. After the process observe the inner compartment containing mostly empty shells and unhatched cysts.

Remarks

The cylindrical separator box described above is very useful at laboratory scale but cannot be sucessfully upscaled for application with large volumes of hatching suspension. As soon as the dimensions of this apparatus become large (for example more than 1 metre in diameter) light penetration in the mixed suspension of nauplii, cysts and hatching debris is limited and as a consequence phototactic separation is ineffective.

For large hatching volumes it is advisable to separate the nauplii in transparent funnel shaped containers using light attraction at the bottom and high salinities to increase density differences consequently favouring the floating of the hatching debris.

6.2.7 Practical procedure

- Upon completion of hatching, filter off nauplii + hatching debris in 120 micron filter bag.
- Wash excessively with seawater (to remove bacteria, glycerol and small dirt).
- Resuspend nauplii + hatching debris in transparent (at least bottom part) container in high salinity water of 50 to 100 ppt, depending on floating (=density) properties of hatching debris.
- Aerate suspension from bottom for about 15 minutes to acclimate nauplii.

- Stop aeration, illuminate bottom part of the container as to attract nauplii.
- After 10 minutes, siphon off nauplii from bottom, collect on filter and wash excessively as to remove high salinity water.

Some strains of *Artemia* can hardly be separated following any of the above mentioned techniques (for example strains that are not phototactic). With these cysts in particular and whenever contamination with empty shells has to be completely avoided, the use of decapsulated cysts is advisable.

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7

CYST DECAPSULATION

7.1 Importance of Decapsulation

While providing as food, complete separation of *Artemia* nauplii from their cyst shells is not always possible and empty shells having a very high bacterial load will cause deleterious effects when ingested by the crustacean predator. Further, about 30% energy of the embryo is utilized only for hatching process. The hard shell or chorion of *Artemia* cysts will be removed without affecting the viability of the embryos by short exposure of the hydrated cysts to a hypochlorite solution, a process that is called cyst decapsulation. The decapsulation technique disinfects the cysts and furthermore, decapsulated cysts can be directly used as food to predators. For example, the larvae of *Lebistes, Macrobrachium, Penaeus, Portunus, Scylla* and *Xiphonophorus* have been successfully reared on a diet of decapsulated cysts. As it eliminates hatching and separation of empty cysts, thereby reducing manual labour, feeding with decapsulated cysts gains importance.

7.2 Decapsulation

7.2.1 Materials

Cysts of Great Salt Lake *Artemia* strain.

7.2.2 Reagents required

Liquid bleach, (NaOCl), 40% NaOH, 0.1 N HCl and saturated brine.

7.2.3 Apparatus required

Ice, refractometer, 120 μm screen, thermometer and conical container.

7.2.4 Decapsulation procedure

Take the dry cyst in a conical container. Hydrate them for 1 to 2 hours in fresh water or sea water (of maximum 35‰). Provide moderate aeration to ensure complete hydration.

Prepare the decapsulation solution with NaOCl, 40% NaOH and 35‰ seawater (For 1 gm of cyst 0.5 g active product and 14 ml of decapsulation solution are needed. 0.33 ml of 40% NaOH is required for 1 gm of cysts).

Transfer the cysts to the decapsulation solution as soon as they have been fully hydrated.

Stir the cysts (by strong aeration or manually) in the decapsulation solution.

Note the rise in the temperature.

Keep water bath to cool the process and don't allow the temperature to raise beyond 40°C (by adding ice, if necessary).

Periodically check the condition of the eggs by observing the colour change under microscope (The colour changes from dark brown to grey and then to orange).

Stop the treatment when the temperature ceases to rise further.

Don't keep the cysts in decapsulation solution for more than 15 minutes.

Filter the cysts from the decapsulation fluid on the 120 μm screen and wash the cysts thoroughly in tap water until no chlorine smell and foaming persists.

Dip the cysts twice in the 0.1N HCl solution.

Subsequently wash the cysts in tap water.

If required, feed the hydrated decapsulated cysts with profound aeration to predator.

For storing the decapsulated cysts, dehydrate the cysts in brine solution for minimum 3 hours.

Replace the old brine with fresh brine and store in polythene containers at 0–4°C in refrigerator.

7.2.5 Principle of the procedure

The decapsulation procedure involves the following consecutive steps:

a. Hydration of the cysts,
b. Treatment in hypochlorite solution,
c. Washing and deactivation of chlorine residues and
d. Either direct use as feed or dehydration for storage.

a. Hydration

Complete removal of the chorion can only be performed when the cysts are spherical in shape and to obtain this desired stage, the cysts are allowed to swell by hydration. In most strains full hydration is reached after 1 to 2 hours exposure to freshwater or seawater (maximum 35‰) at 25°C. Prolonged hydration will induce the embryological development in cysts and hence care is to be taken to process the cysts at the end of 1 to 2 hours hydration.

b. Treatment in hypochlorite solution

Either liquid bleach, NaOCl, or bleaching powder, $Ca(OCl)_2$, can be used to prepare the decapsulation solution. When NaOCl is used, sodium and OCl^- become ionised in solution and HOCl

is formed in water. When $Ca(OCl)_2$ is used, two ions of OCl^- are produced for every molecule of hypochlorite. It is believed that OCl^- acts on the chorion but it is still not definite. The activity and concentration of OCl^- are maximum at pH 10. 0.5 g active product and 14 ml of decapsulation solution are required for each gram of dry cysts to be decapsulated. In many countries, $Ca(OCl)_2$ is a cheaper source of active chlorine than NaOCl. $Ca(OCl)_2$ is a much more stable product than NaOCl and can be stored for longer periods. The activity of $Ca(OCl)_2$ is usually correctly mentioned on the label of the commercial products (mostly 70% weight percent activity). The activity of NaOCl solution can eventually be verified and determined by measuring the refractive index with a refractometer (when available) by using the following formula.

$$Y = 3000\ x - 4003$$

Where Y = activity of NaOCl in gm per litre and x = refractive index. With NaOCl, 0.15 g technical grade NaOH (0.33 ml of a 40% solution) has to be added per gram of cyst to raise the pH of the decapsulation solution to about 10. In the case of $Ca(OCl)_2$, 0.67 gm of Na_2CO_3 or 0.4 gm of CaO has to be added. Decapsulation solution has to be made up with 35‰ seawater. In the case of $Ca(OCl)_2$ solution, it is critical to first dissolve the bleaching powder and only then add CaO or Na_2CO_3. After thorough mixing (about 10 minutes with strong aeration), the suspension has to be allowed to settle down and only the supernatant $Ca(OCl)_2$ solution should be used.

After transferring the cysts to the decapsulation solution, they have to be kept in suspension by manual stirring or continuous aeration. Within a few minutes, the exothermic oxidation reaction starts, foam develops and as the chorions dissolve, a gradual colour change in the cysts will be observed from dark brown to grey and then to orange. During decapsulation, the temperature has to be checked regularly and ice has to be added in order to prevent the raise in temperature above the lethal level of 40°C. Prolonged immersion in the decapsulation solution will kill the embryo and hence the cysts have to be removed from the solution as soon as

the process is over. The completion of the process can be judged by periodically observing the colour change in a few eggs under microscope. Besides, when the process is over, there will be no more increase in temperature.

C. Washing and deactivation of chlorine residues

During treatment in decapsulation solution, HOCl acts on the chorion of the cysts and as a result of the above action, some organo-chlorine compound is formed which gets adhered to the decapsulated cysts and reduces the keeping quality and utility of the decapsulated cysts. Hence, after washing, 1% $Na_2S_2O_3$ may be added at the rate of 0.5 ml/gm of cyst, which forms a soluble compound with the organo-chlorine compound, thus removing them from the decapsulated cysts. This deactivation method with thiosulphate is, however, not entirely satisfactory because upon long-term storage of decapsulated cysts at high densities, the hatchability decreases. A tentative explanation is that the saponification layer formed around the embryos and the chlorinated compounds trapped into it, are not entirely deactivated by the thiosulphate. However, higher viability (upon storage in brine) than the manipulation with thiosulphate is at present achieved by treating the decapsulated cysts with 0.1 N HCl after washing out the hypochlorite solution. At first, the decapsulated cysts are filtered from the hypochlorite solution and washed on the 120μ m screen with tap water until no more chlorine smells and no more foaming persists. Secondly, the cysts have to be dipped a couple of times in 0.1 N HCl. Subsequently, the cysts are again washed with tap water or seawater.

D. Direct use or dehydration for storage of the decapsulated cysts

The hydrated decapsulated cysts can be offered directly as food source to the predator. If needed, they can be stored for a few days in the refrigerator at 0–4°C. When used directly as food, it is critical to assure sufficient aeration and circulation to keep the cysts in suspension for better feeding by predators, as the hydrated decapsulated cysts sink in seawater or freshwater.

For storing the decapsulated cysts, dehydration has to be done after completion of deactivation and washing procedures. For this, the cysts have to be kept in NaCl saturated brine (±330 g/l). After about 3 hours, the brine has to be renewed for effective dehydration. When dehydrated, the decapsulated cysts become coffee-bean shaped and sink, even in saturated brine solution. These dehydrated decapsulated cysts have to be drained off on a 120μm screen, transferred in plastic containers, topped with fresh brine and stored in the refrigerator or freezer.

The storage of decapsulated cysts in brine has nonetheless its limitations. During the first six months after decapsulation the cysts keep their maximum hatchability, even when stored at ± 20°C. Over longer periods, cysts viability appears to decrease as a function of storage temperature. The decreased hatchability of decapsulated cysts stored in brine is probably due to their relatively high water content (about 20%). When the water content ranges from 10 to 35%, indication of enzyme activity and a slow but significant drop in the ATP concentration have been reported.

Long term storage of dried decapsulated cysts (water content below 5%) is possible when they are kept in a dry and oxygen free medium (nitrogen flushed or vacuum sealed containers).

7.2.6 Observation

Observe the change in cyst colour from dark brown to grey and then to orange, during decapsulation. Observe the sinking of decapsulated cysts in brine. Observe the coffee-bean shaped structure of the dehydrated decapsulated cysts; observe hatching in decapsulated cysts: breaking stage, hatching stage, transparent chorion.

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8

QUALITY OF *ARTEMIA* CYSTS FOR USE IN AQUACULTURE HATCHERIES — QUALITY IMPROVEMENT OF *ARTEMIA* NAUPLII THROUGH ENRICHMENT

8.1 Quality

The quality of *Artemia* cysts as naupliar food source for fish and/or shrimp larvae is determined by various characteristics:

A. Hatching performance: Amount of nauplii produced per gram of cysts expressed as Hatching Efficiency (*i.e.* number of nauplii per gram cysts; best products yield 300,000 nauplii/gram) and/or as Hatching Output (*i.e.* mg dry weight naupliar biomass per gram cysts; best products yield about 600 mg nauplii/g). Rate and synchrony at which nauplii can be hatched expressed as Hatching Rate) (best products start to hatch after about 15 hrs since incubation in natural seawater at 25°C and yield 90% of their maximum hatchability within another 5 hrs).

B. Size of the freshly hatched nauplius (instar I stage) which in function of the strain of cysts used fluctuates from 428 upto 517 microns. As long as nauplius size does not interfere with the ingestion mechanism of the predator, the use of larger nauplii (with a higher individual weight) will be beneficial as the

predator will spend less energy in taking up a smaller number of larger nauplii to fulfill its food requirements.

C. Nutritional value of the nauplii which appears to be predator-specific. However, the following generalisations can be made:

Artemia can be contaminated with high levels of heavy metals and/or chlorinated hydrocarbons. Feeding the predator larvae with contaminated *Artemia* can result in increased mortality levels (acute toxicity) or in bioaccumulation of toxicants which only during periods of stress (*e.g.* lipid consumption during weaning on artificial diets) will result in increased sensitivity/mortality (indirect chronic toxicity).

Contaminated *Artemia* can, however, be safely used for biomass production (intensive culturing) since per unit of biomass weight, the contamination levels will decrease by a factor 500 times in the adult brine shrimp.

The fatty acid pattern, more particularly the content in poly-unsaturated fatty acids (PUFA's) appears to vary in the *Artemia* as a function of the source of the cysts and even within a particular source (strain) from cyst harvest to harvest. This is especially critical for marine predators since they apparently need high levels of the fatty acids 20:5ω3 and 22:6ω3 (the latter being seldom present in detectable levels in *Artemia* nauplii): *i.e.* with PUFA-poor *Artemia* low survival is reported for marine fish and shrimp either in the hatchery phase or only in the later nursery or growout phase when the animals have already been switched for some time to another food source (provided a more sensitive criterium than "survival rate" could be evaluated, it is highly likely that at the end of the hatchery phase differences in overall physiological condition could already be noticed between fish/shrimp fed PUFA-rich versus PUFA–poor *Artemia*).

The reason for the poor PUFA-profiles in some (most) *Artemia* cyst sources is related to the biochemical composition (*i.e.* PUFA-content) of the (sometimes changing) natural food of *Artemia*. In

fact in the high salinity environment where *Artemia* live, those species of diatoms and flagellates, known to be rich in PUFA's and being the food for the natural zooplanktonic diet of marine fish and shrimp (*e.g.* copepods, rotifers, etc.) do not or only seldom occur.

In this regard it is advisable to check the PUFA-quality of a new source (and even batch) of *Artemia* cysts by gas-chromatographic analysis. When the quality is poor or in any case where such sophisticated analysis cannot be performed, freshly hatched *Artemia* nauplii should only be used for a short period of time, *i.e.* until 1 to 2 day old *Artemia* metanauplii that have been enriched with PUFA's via their diet can be offered to the predator larva.

Different particulate products can be used for enrichment of the instar II *Artemia* nauplii through bioencapsulation and/or metabolisation (accumulation), *e.g.* microencapsulated diets and micronized particles containing respectively coated with PUFA-rich oils such as cod liver oil; PUFA-rich oil emulsions; or micro-algae rich in PUFA's such as the diatoms *Chaetoceros, Skeletonema,* and the flagellates *Isochrysis, Nannochloris.* Diatom cultures being easily produced on a large-scale using cheap fertilizers, it is advised to select the latter algal cells (eventually after breaking down into single cells or smaller chains by heavy aeration treatment, as to facilitate uptake by *Artemia*) as enrichment diets for the *Artemia* nauplii.

Optimal enrichment results are achieved by feeding the *Artemia* nauplii in high densities (25 - 50,000 nauplii per 1) at water temperatures of 25 - 30°C during 24 to 48 hrs post-hatching in dense concentrations of the PUFA-rich algae or particle suspensions (100 – 500,000 cells/ml). It is clear that optimisation of naupliar enrichment techniques only can be performed through comparative gas-chromatographic analysis of the PUFA-contents of the cultured *Artemia* metanauplii.

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9

ARTEMIA PRODUCTION IN TEMPORAL SALTPANS

9.1 Introduction

In the tropical-subtropical belt thousands and thousands of hectares of saltponds are in operation during the dry season. When monsoon starts, salt production must be abandoned and the ponds are often converted into paddy fields or fish/shrimp ponds.

This type of salt making is very primitive and is often run at limits of profitability. In many countries (*e.g.* Thailand, Panama, Costa Rica) hundreds of these family-operated salt farms are being abandoned for socio-economic reasons. During the past 5 years it has been demonstrated in Asia (*e.g.* Thailand, Philippines, Burma, Vietnam) and Central America (Costa Rica, Cuba) that through proper pond modification and biological management temporal saltponds can produce an extra source of income under the form of *Artemia* cysts and biomass. Furthermore it appears that salt purity is being improved through the presence of *Artemia* earlier in the brine system. This salt-cum-*Artemia* production is not only attractive from the socio-economic point of view, local aquaculture activities furthermore can profit from the availability of cheap *Artemia* products.

9.2 Pond Modification

Temporal saltponds are mostly operated at water depths of not more than 10 cm; as a consequence water temperatures easily

run up to over 40°C which is lethal for *Artemia*. Furthermore shallow waters promote the development of phytobenthos which in view of its size cannot be used as food by the *Artemia*. Only the ponds with salinities in range of 100 to 150 ppt should be

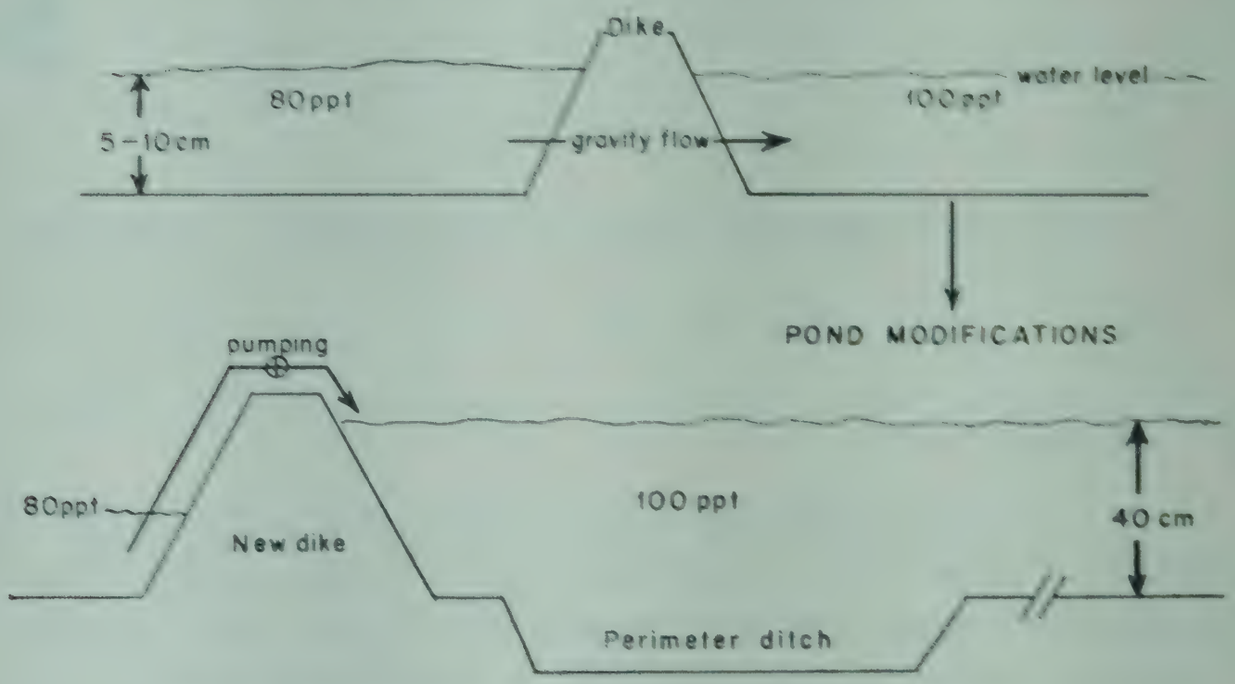

Fig. 8. Conventional salt ponds after pond modification.

deepened for *Artemia* production integration. Water depths should be 40–50 cm minimum, 50–70 cm by preference. Excavation of the ponds being too expensive the cheapest way is to increase dike heights using the soil from a perimeter ditch (Fig. 8).

9.3 *Artemia* Inoculation — Biological Management

The basic principles for an *Artemia* inoculation in a temporal saltwork are as follows:

a. introduction of a small quantity of freshly hatched nauplii (1 to 10 per litre pondwater) as early as possible in the brine circuit where no more predators (fish, crustaceans as well as inflying insects such as Corixidae) are being found (mostly at salinity levels around 100 ppt);

b. through proper fertilisation (food availability) maintain a dominant ovoviviparous mode of reproduction as to assure a fast

increase in population density (100 and more individuals per litre);

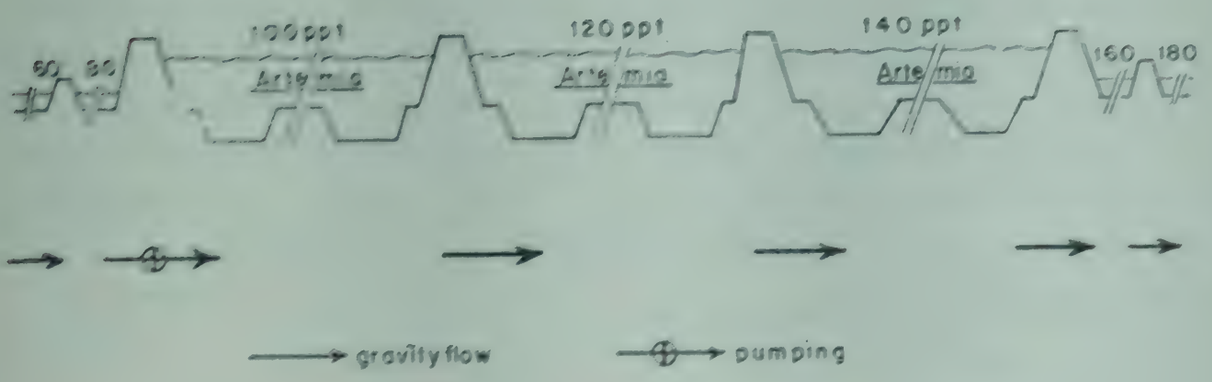

Fig. 9. Schematic cross section through modified saltwork.

c. start biomass harvesting (adjust rate in function of productivity), maintain proper fertilisation rates as to assure continuous recruitment through ovoviviparity or

d. expose *Artemia* to higher salinity levels and/or increase fertilisation rates as to provide extra stress to the population for induction of oviparity, maintain proper fertilisation rates for continued productions.

9.3.1 Inoculation procedures

- select suitable strain of cysts in function of availability, temperature resistance, size of cysts/adults or other characteristics;

- in function of hatching quality data of available cysts (hatching rate and efficiency) calculate quantity of cysts needed as to produce enough nauplii for inoculation;

- incubate cysts under optimal hatching conditions (seawater, light, pH, etc.) in the laboratory or under field conditions (*e.g.* battery-operated air pumps); start incubation as to reach harvesting time in late afternoon;

- harvest nauplii in the instar I stage (this is essential as the later stages do not have the same physiological resistance to salinity stresses) and transfer animals as soon as possible in the inoculation pond (no temperature nor salinity acclimation needed). For long transportations (hours) animals should be filtered over 150 μm screen, thoroughly washed to eliminate bacteria and be incubated in cold (0 - 4°C) seawater at densities of 5 up to 10 million nauplii per litre; slow aeration or continuous shaking has to be provided as to prevent suffocation of the motionless animals.

9.3.2 Fertilisation procedures

Productive intake waters of saltwork (*e.g.* from mangrove area or from estuary) might provide enough food for the *Artemia* at the start, soon however, food densities will be too limited to maintain a dense *Artemia* population.

Prior to start the inoculation, ponds should be fertilized either with inorganic agricultural fertilizers or preferentially with cheap organic manures such as chicken excreta. Only apply fertilisation when ponds are at maximum water depth as to assure bloom of phytoplankton (try to prevent the development of phytobenthos as it is not available as food source for the *Artemia*). Secchi-disc (turbidity) measurements should be made as to evaluate food concentrations in the pond water (optimal levels around 20 cm); weekly up to bi-weekly dressing should be applied in function of fluctuations in water-turbidity. Examples of fertilisation rates (tropical situation) per hectare pond water are given below.

- *inorganic fertilisation:* At start 100 to 200 kg mono-ammonium-phosphate (NPK–ratio: 16–20–0) together with 50 to 100 kg ammonium-nitrate (30–0–0); weekly dressings of 50 kg and 25 kg respectively. Good results have also been reported when using 25 – 50 kg di-ammonium-phosphate (18–46–0) and 40 kg urea (44–0–0); weekly dressing of 15 kg and 10 kg respectively.

- *organic fertilisatina using dry chicken manure*: At start 500 to 1000 kg per hectare; bi-weekly dressing of 150 to 250 kg.
- *When pH of water is below 8.0*: add lime (CaO) at a rate of 500 kg per hectare.

9.3.3 Culture maintenance — biological management

- Keep daily records of following parameters:

  minimum – maximum water temperature, rainfall, salinity levels, water turbidity, general population conditions

- Once upto twice a week measure pH and evaluate animal densities through visual estimation using submerged white-painted plate-surface (determination of population density through sampling procedures is useless in view of strong heterogenic distribution of brine shrimp). Take population samples from each pond and analyse population composition; consider 4 size-classes: nauplii, juveniles, pre-adults and adults; changes in population composition can be correlated with the overall production status of the population, *e.g.* presence of only adults reflects status of no recruitment as a result of either food limitation or dominant cyst reproduction. Analyze reproduction status of adult specimens by observation of their brood sac contents: full, empty, presence of nauplii or cysts, activity of brown shell glands.
- In view of observations made, adjust harvesting rates, fertilisation rates, salinity regimes, etc.

9.3.4 Procedures for harvesting and processing of cysts and/or biomass

See Chapter on 'Exploitation of *Artemia* from natural habitats'.

9.3.5 Quality control

Product quality, especially with regard to the biochemical composition of cysts and adults (*e.g.* fatty acid profile) will be determined to a large extent by the food composition in the ponds.

When poor quality is prevailing dominant presence of other types of algae, bacteria and/or organic detritus could be tested (induced) by using other fertilizers or fertilizer-combinations (*e.g.* combined organic and inorganic fertilisation).

It should be clear, when the intake waters of the salt operation are contaminated with pollutants (*e.g.* chlorinated hydrocarbons, heavy metals) that these products eventually will be bio-accumulated in the *Artemia* biomass and consequently in the cysts. In this regard proper site selection and/or adjustments in water intake (*e.g.* at low tide or high tide, from sea or estuary, etc.) should be considered carefully.

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10

ARTEMIA PRODUCTION IN CONTROLLED SYSTEMS

10.1 Introduction

Artemia grows from nauplius to adult in 2 weeks, increasing in length by a factor of 20 and in biomass by a factor of 500, thereby offering very high rate of production within a short period. *Artemia* adults and preadults form an ideal food for prawn/fish juveniles in their nursery phase. They can be intensively cultured under controlled conditions in the same fish/prawn nurseries in order to have a ready availability of feed for juveniles and hence *Artemia* biomass production under controlled environment gains more and more importance.

The ideal *Artemia* culturing technique suitable for large scale application in controlled environment implies the following prerequisites:

a. Good oxygenation of the medium to allow culturing at high density (thousands of animals per litre).

b. Continuous circulation of the medium to maximize food availability to the brine shrimp which are swimming continuously.

Biomass production under controlled conditions (intensive culture of *Artemia* from nauplius to adult stage) can be carried out

either in batch or in flow-through culture systems. In batch culture, no water change is taken place whereas in the flow-through culture system, the culture water will be renewed continuously with consequent removal of all particulate and dissolved metabolities.

10.2 Batch Culture System

Of the various techniques, tested for high density culturing of *Artemia* larvae from nauplii to adult in batch system, the air-water-lift (AWL) operated receway, originally described by Mock to culture benthonic postlarval *Penaeus,* proved to be the most suitable. The construction and operation of the culturing tank is indeed simple. Furthermore it has been proven that high survival, acceptable growth rate and high production results can be obtained in this method.

10.2.1 Equipments required

Air-water-lift raceway; plate separator; filter screen system with filter bags of 200, 250, 350, 450μ m; transparency stick; pH meter and balance.

10.2.2 Material required

Rice bran as feed.

10.2.3 Construction of the Air-water-lift (AWL)-raceway (Fig. 10, 11)

An *Artemia* raceway essentially consists of a rectangualr tank with a central partitioning. It will be better if the corners of the tank are curved, eventhough it is not absolutely necessary. The distance between the central partitioning and the lengthy side of the tank is called the channel width. In order to obtain an optimal water circulation, the central partitioning should not be closer to the wall of the small side than 1 to 2/3 of the channel width. The partitioning should also be kept 2 to 5 cm off the bottom of the tank either by suspending it from two or more wooden bars resting

on the sidewalls of the raceway or by keeping it in its central position on top of small blocks. The most important parameter for the configuration of the tank is the height/width ratio which should

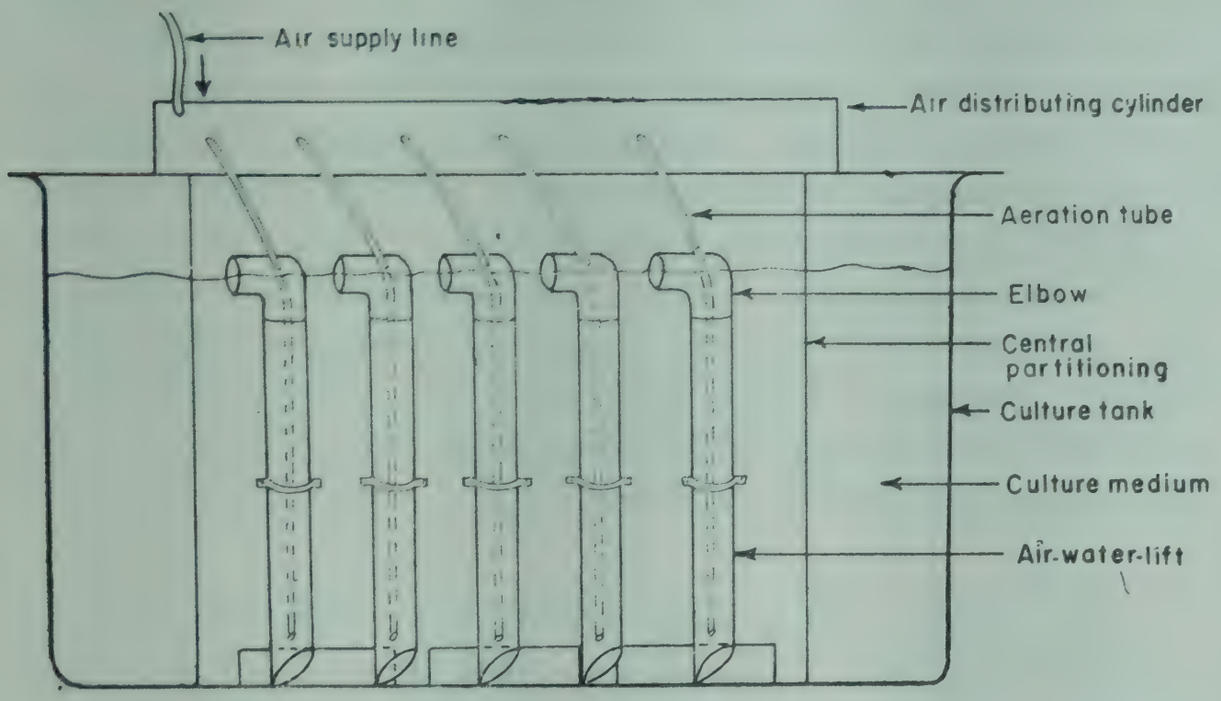

Fig. 10. Raceway.

be kept close to 1. For optimal water circulation, using axial blowers, the water depth should not exceed 1 metre. Various materials such as concrete, marine plywood and fibreglass, can be used to construct receway tanks.

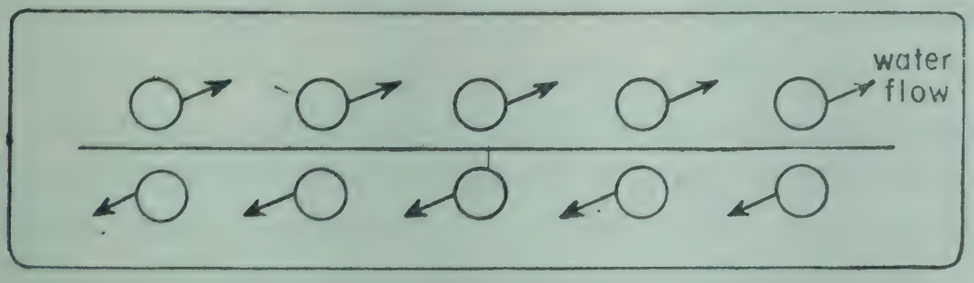

Fig. 11. Top view of raceway.

The easiest way to construct an AWL is to use PVC pipes and elbows. Depending on the materials available, various systems such as plastic tubing with screw, PVC-ring with screw and piece of plastic or wood with rubber band, can be considered to attach the AWL's to the central partitioning to keep them in a well defined

position for an optimal water circulation in the raceway. For this purpose, the elbow outflows should make an angle of 30 to 45° with the central partitioning. Although the oxygenation of the culturing medium is maximal when the AWL's outflow extends a few centimetres above the water surface, it is always better to keep the outflows of the AWL's half submerged to prevent formation of tiny air bubbles. It has been observed that the ingestion of small air bubbles by the brine shrimp or their trapping between the thoracopods makes the animals float and leads to mortality. For the same reason the airstones will not be made use of although it is well known that these can greatly aid to oxygenate the culture medium. Airstones furthermore cause foam formation which again can result in mortality because brine shrimp trapped in the foam cannot get back in the water.

With regard to the number of AWL's per tank, optimal circulation and aeration is obtained with pipes installed at 25 to 40 cm intervals. In order to assure the maximal water-lift-effect, it is clear that the diameter of the AWL's should be related to the water depth. If the water level is 40 cm, the inner diameter of the AWL should be 40 mm which will provide 6.6 litre/minute/AWL of air to displace 12.5 litre/minute/AWL of water. (If the water level is 20 cm, the inner diameter of the AWL should be 25 mm to provide 2.7 litre/minute/AWL of air which will displace 4 litre/minute/AWL of water).

Small polyethylene tubing (3–6 mm in diameter) will serve as aeration line and it can be mounted in the AWL through a hole at the top of the PVC elbow. The aeration line can be raised or lowered at will and its tight fitting through the elbow prevents any undesired displacement. To assure the best water-lift-effect, the aeration lines should extend as deep as possible in the AWL. All the aeration lines will take lead from a central air distributing container so as each aeration line need not have a separate regulating valve system. An identical and constant aeration is obtained by adjusting all air tubes to the same hydrostatic depth in the AWL's.

Plate separator (Fig. 12)

The plate separator is a rectangular tank (50 cm length x 30 cm width x 20 cm height) which is divided into three inter-connected compartments by two perforated vertical sheets

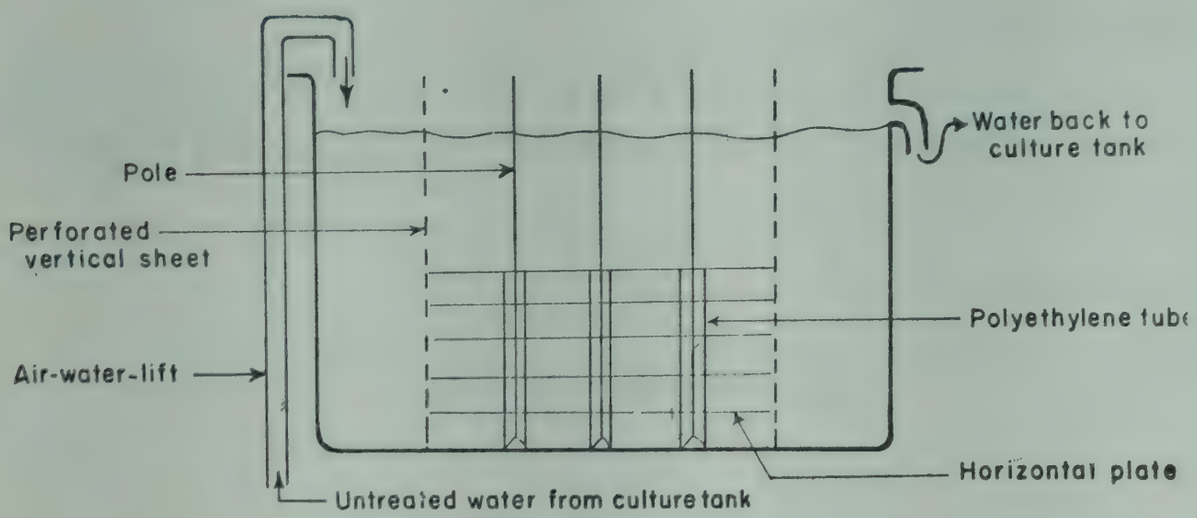

Fig. 12. Plate separator.

(30 cm width x 20 cm height x 0.2 cm thickness). In the middle compartment, plates (five in number, each one of 30 cm length x 30 cm width x 0.2 cm thickness) will be mounted horizontally on the poles one above the other at specific intervals (2.5–3 cm) with the help of removable polyethylene tubes. The water from the culture tank will be pumped to the first compartment by AWL. The water will pass through the plates and leave the separator from the last compartment after a retention time of about 20–30 minutes, during which faecal pellets, exuviae and other larger particles will settle down in the bottom and on the plates. It is better if the plates have a coarse surface.

Filter-Screen System (Fig. 14)

The filter-screen system is made of a filter-frame over which the filter screen bag is tied. An air collar is attached to its bottom. The filter frame can be made out of wood or PVC tube and the screen bags are of nylon material with required mesh size. The width in the upper part of the filter frame (15 cm) is more than that

of the lower part (10 cm) and the length at anterior end (45 cm) is considerable more when compared to the bottom length (20 cm). This special shape helps to produce a continuous curtain of air bubbles against the sides of the filter screen bag which will prevent clogging during operation.

Transparency stick (Fig. 13)

It is a graduated stick of about half a metre in length to which a disc is attached at one end. On the disc, letters will be painted

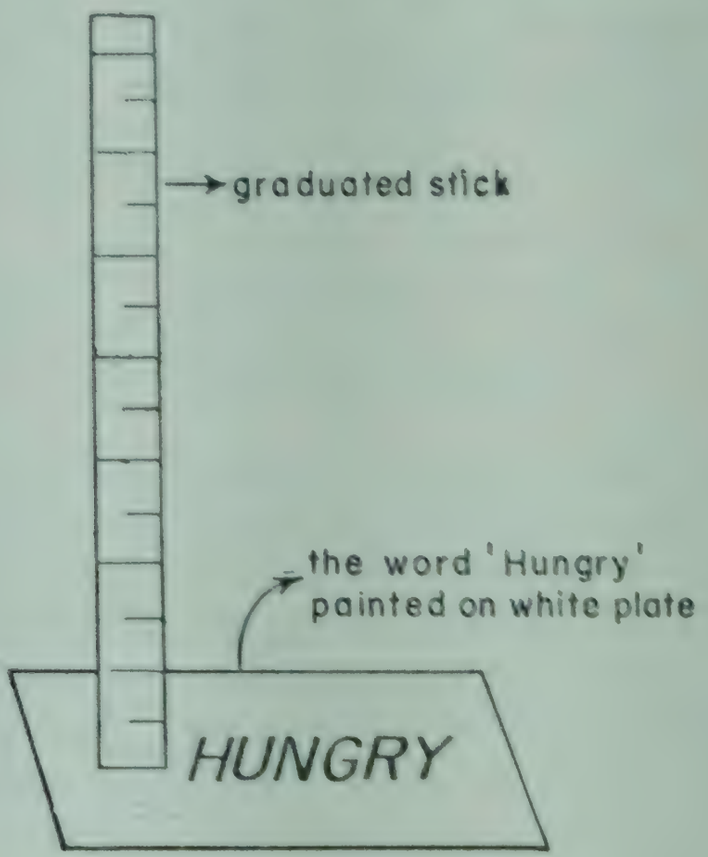

Fig. 13. Transparency stick.

(for example hungry). It is a type of Secchi disc and the transparency of the culture medium is measured by submerging and lowering the stick in the culture medium until the letters will be just visible.

10.2.4 Procedure

1. Fix the raceway in the culture tank.
2. Check whether all the AWL's are working properly by pumping required seawater.

3. Adjust the angle of the AWL's to get a desirable flow direction.
4. Discard the hatching solution by retaining the nauplii on 100 μm screen and wash the nauplii with fresh seawater.
5. Gently introduce the nauplii instar I to the culture medium. (Stocking rate varies from 3,000 to 10,000 nauplii per litre according to the rate of feeding and water management).

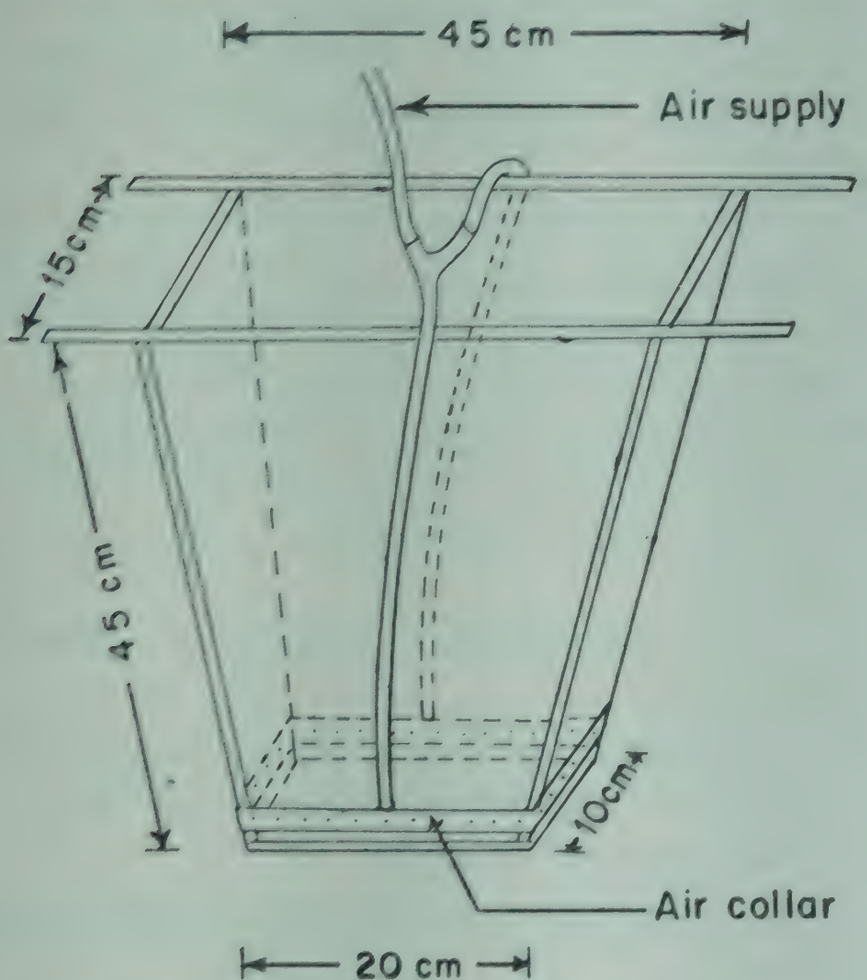

Fig. 14. Filter frame for Filter-Screen System.

6. Stock the tank in the evening to avoid the prevailing high temperature during day time.
7. Start feeding on the next day morning onwards.
8. Prepare the feed (rice bran) as follows:
   a. Choke the feed in fresh water; eventually grind it with coarse salt in a kitchen blender.
   b. Squeeze the feed through 50 μm screen.
   c. Collect the feed solution in a bucket.
   d. Vigorously aerate the feed solution for about 1–2 hours.

e. Stop aeration and allow the feed particles to settle for about ½ hour.
f. Discard the supernatant solution.
g. Take the sediment and make a solution with sea water and it can be utilized for feeding.

9. Assure food is distributed several times a day.
10. Monitor the feed distribution by measuring the transparency with a transparency stick. (If the stocking rate is 10,000 nauplii per litre, the transparency should be maintained at 15–20 cm).
11. Fix the filter screen system and the plate separator for treating the culture medium. (If plate separator and the filter screen system are not available, remove sediment twice daily, one at morning before feeding and one at evening).
12. Make sure that the air collar of the filter screen system is in proper position so that air comes equally from all directions.
13. Fix the AWL of the plate separator in the centre of the filter screen system.
14. Take care that the water should flow to the culture tank from separator.
15. Change the filter screen bag as the animals grow in size *i.e.* 200 μm, 250 μm, 350 μm, 450 μm respectively.
16. Daily clean the filter screen bag.
17. Once in alternate days clean the plate separator by dismantling the plates.
18. Periodically check the pH and dissolved oxygen. (If pH drops below 7.5, add sodium bicarbonate (0.3 to 1.0 g/1). If oxygen level goes below 2 mg/1, change half of the culture medium with fresh sea water).
19. Frequently take sample and observe the condition of the growing brine shrimp under microscope.
20. The biomass has to be measured by taking the wet weight of the growing *Artemia* present in one litre of the culture medium (by retaining *Artemia* in a sieve and weighing in a balance).
21. Harvest the grown up *Artemia* by draining culture over 450 μm filter or in case of high density culture by simply cutting off

the aeration for half an hour and subsequently scooping out *Artemia* which have come up to the surface.

10.2.5 Principle of the procedure

Raceway system with AWL yields the following results:

a. Continuous aeration.
b. Almost homogeneous circulation of the medium.
c. Keeping all particulate matter in suspension.
d. Uniform distribution of the added feed within a short time.

Batch culture in raceway system can be carried out with the culture medium of any salinity. However, for easy accessibility, sea water will be used and if the culture medium gets frequently contaminated with ciliates and other competitors and predators, it is safer to do biomass production in 50–100‰. Stocking will be effective if it is done at instar I stage as *Artemia* instar I nauplii will remarkably tolerate salinity stress by sudden change to any salinity. Freshly hatched nauplii should be stocked after removing them from the hatching solution and subsequently washing with fresh sea water. Heavy bacterial load will be present in the hatching solution and hence care should be taken to completely wash the nauplii with fresh sea water. Rate of stocking depends upon the feed availability and water management and it can be safely increased to 10,000 instar I nauplii per litre if feeding is maintained at 15–20 cm transparency. As *Artemia* is a non-selective filter feeder, it can be cultured by feeding them with a wide range of feed, both live and inert materials. However, the particle size of the feed is very important and it should be less than 50 μm. Hence, the feed should be squeezed (if inert feed) or passed (if algal feed) through 50 μm sieve.

Soluble products are not taken up by *Artemia* and they will be decomposed by bacteria in the culture medium thereby deteriorating the water quality by gradual build up of toxic substances such as ammonia. Hence, the feed should be properly prepared to get rid off the dissolved matter. This can be achieved by aerating the feed solution for 1–2 hours and allowing the feed particles to settle

by cutting off the aeration for ½ hour. Dissolved matter will be in solution and it will be discarded. *Artemia* will go on continuously filtering the culture medium and hence medium must contain adequate food at all times. Hence, food distribution is very important. It is noted that transparency of the culture medium is found to be a very useful parameter for determining the food level present in the medium. If the stocking rate is about 10,000 nauplii per litre, feeding should be continued until the medium reaches a transparency of 15–20 cm. By providing regular food distributions optimal water quality can be maintained.

As the animal grows, *Artemia* moults and the exuviae and the faecal pellets hamper the food uptake by *Artemia*. Besides, they affect the water quality. These particulate wastes should be continuously removed from the culture medium from day 4 onwards. This primary water treatment can be performed with filter screen system and plate separator. The water to be treated will be pumped to the plate separator by AWL and at a retention time of 20–30 minutes, the larger particles, faecal pellets and exuviae settle down at the bottom of the separator and on the plates while water with small food particles in suspension drain back into raceway. In order to assure that *Artemia* is not pumped to the plate separator, a filter screen system with aeration collar is used. The air collar reduces the clogging by raising a continuous curtain of air bubbles against the sides of the filter bag. However, it is better to clean the filter bag daily with a jet of water (tap water) to have an effective prevention from clogging. As the animals grow, the filter bags should be changed by new ones with progressively larger mesh width.

When inert products will be given as feed, the pH of the culture medium will go down and if it goes below 7.5, the pH should be raised by adding $NaHCO_3$ at the rate of about 300 gm per ton of water. Frequent observation of *Artemia* under microscope will enable a person to judge the progress of the culture. Well fed *Artemia* will have full digestive tube. Further, *Artemia* will not feed when its mouth is blocked by the sticky nature of the feed provided (for example rice bran). Similarly, *Artemia* has to starve

when its filtering surface of the thoracopods are covered by larger waste particles. These adverse conditions will be clearly detected when an observation is made under microscope.

The growth of the animal should be periodically checked by estimating the biomass. Biomass can be calculated by taking wet weight of the *Artemia* present in a litre of medium and calibrating to the total tank volume. The culture period varies with the temperature and the strain selected. Generally, *Artemia* will reach a harvestable size within 2 weeks time (measuring about $\pm$ 8 mm in total length).

Harvesting of dense cultures is facilitated by taking advantage of the special surface respiration behaviour in *Artemia*. When the aeration is cut off, the oxygen concentration drops to a critical minimum after about 30 minutes and the *Artemia* concentrate in dense numbers at the water surface from where they can be easily scooped out with a net.

By batch culture system, biomass production amounts to an average value of 5 kg/cubic metre of water when stocking is done at 10,000 nauplii per litre and food distribution at 15–20 cm transparency.

10.3 Flow–Through System

More intensive *Artemia* culture can be achieved with flow-through culture system in which continuous renewal of culture water will be maintained. In all other aspects, it resembles the batch culture system.

10.3.1 Equipments required

Air-water-lift raceway; filter screen system with filter bags of 130, 200, 350, 450 μm; transparency stick; pH meter and balance

Feed: rice bran or algal culture.

10.3.2 Procedure

1. Set up the raceway.
2. Fix the filter screen system with 130 μm filter bag.
3. Siphon off the water from the tank via filter screen system.
4. Maintain a continuous inflow of fresh culture medium (with loaded food - for example algae or rice bran suspension), to the culture tank.
5. Keep the retention time at 4 hours in the initial stage.
6. Maintain the transparency level at optimal levels by (semi) – continuous food addition (or when using algae by checking the cell concentrations).
7. Take the transparency readings only from inside the filter screen system.
8. Change the filter bag to 200, 350 and 450 μm respectively on day 3, 6 and 9 onwards.
9. Clean the filter bag daily.
10. Daily observe the condition of growing *Artemia* under microscope.
11. Estimate the biomass daily.
12. Maintain the retention time of 1 hour from day 10 onwards.
13. Harvest the reared *Artemia* by initially cutting off the aeration and subsequently scooping out the *Artemia* from the surface.

10.3.3 Principle

In flow-through culture system the medium is continuously renewed as against the batch culture system. This water change results in consequent removal of all particulate dissolved metabolites which in turn leads to high production. An interchangeable and self cleaning screen system (installed in the culture tank with aeration collar) that retains the animals in the culture tank but allows drainage of water and faecal pellets is the important key that makes flow-through culture system technically feasible. As the animals grow, accumulation of metabolites will increase and hence

from day 10 onwards, the retention time should be kept at about one hour.

By this flow-through culture system, 20,000 nauplii per litre can be reared with algal or rice bran feed resulting in production yields of 25 kg/m^3/2 weeks.

10.3.4 Remark

Aside from rice bran other agricultural by-products or waste-products could be evaluated as potential feed for *Artemia*. Critical parameters in the choice of a suitable *Artemia* feed are the solubility in water (should be minimal), particle size (below 50 microns, preferentially in range of 1–20 microns), clogging effect (should not be a sticky product as it can clogg the filter apparatus as well as the mouth, especially in adult *Artemia*) and the quality constancy of the stored product.

It should furthermore be clear that in function of the granulometric as well as the nutritional composition of new *Artemia* feeds, optimal turbidity levels have to be determined (in fact they could be significantly different from the ones reported here for rice bran).

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